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(54) Title: A RAPID, QUANTITATIVE METHOD FOR THE MASS SPECTROMETRIC ANALYSIS OF NUCLEIC ACIDS FOR GENE EXPRESSION AND GENOTYPING

(57) Abstract: The invention provides methods of identifying one or more nucleic acids in a sample. The nucleic acids, for example, expressed genes in a cell, can be identified by contacting the nucleic acids with oligonucleotides having detector tags, and selector tags to form tagged oligonucleotides. Each nucleic acid can be uniquely identified by mass-spectrophotometric analysis of the detector tag.

A RAPID, QUANTITATIVE METHOD FOR THE MASS SPECTROMETRIC ANALYSIS OF NUCLEIC ACIDS FOR GENE EXPRESSION AND GENOTYPING

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. § 119(e) to United States Provisional Application 60/221,479, filed July 27, 2000, the entire contents of which is incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates generally to methods for detecting and measuring the level of nucleic acids, and specifically to detection and measurement methods using mass spectrometry.

BACKGROUND

Although a variety of methods to detect and measure nucleic acids have been developed, no method provides a highly accurate means of detecting many genes in a biological sample. The simplest method in common practice for detection of mRNA transcripts is the northern blot. Northern blot analysis can be used to detect a small number of transcripts of interest, however quantitation of the level of a specific transcript using northern blot analysis is difficult and often inaccurate. RT-PCR can be used on its own or in an intermediate-scale method known as the rapid analysis of gene expression (RAGE). RT-PCR and RAGE suffer from biases resulting from the PCR priming and amplification process and are further limited by the use of measurement of band intensity on gels to quantify gene levels. In addition, the methods are not well-suited to a high level multiplexing, *i.e.*, measuring many genes at once in a single sample.

In order to address the poor quantitation of previous methods, newer methods based on sequencing have been suggested. One such method, serial analysis of gene expression (SAGE), allows the quantitative and simultaneous analysis of a large number of transcripts. In SAGE, the cDNA library constructed from all the transcripts in a cell, i.e., the transcriptome, is concatenated into large chunks and then sequenced. The sequencing data is computationally converted into quantitative levels of gene expression via the frequency of occurrence of sequences representing a given

gene transcript. This method can also be used for gene discovery, but it cannot be targeted to specific subsets of genes of interest (*e.g.*, all known oncogenes or all known tumor suppressors or all known G-coupled membrane receptors). SAGE is accurate, but it is slow and relatively expensive since it requires a large amount of sequencing for each sample to be studied.

One new method, called massively parallel signature sequencing (MPSS), is a high throughput method making use of specially litigated adapters and signature sequencing. It is sequencing-based and suffers many of the same disadvantages as SAGE, but it is more efficient because separation of cDNA fragments is not required.

The most common methods in practice for large-scale analysis of the transcriptome employ DNA microarrays, either robotically spotted or microfabricated. Such arrays permit biologists to monitor 5-10,000 genes per experiment in most implementations, for example, by using a DNA chip, provided that the experiment begins with large amounts of starting sample. Thus, RNA extraction must be from sizable amounts of tissue, cell or embryo cultures, or that the initial RNA sample must be amplified via various PCR-based strategies. It would be highly desirable to eliminate amplification steps and to instead make measurements of RNA presence and levels directly.

Methods using microarrays suffer various limitations. First, microarrays rely on hybridization to cDNAs or oligonucleotides that are bound to the surface of a solid support. The kinetics and physics of such interactions are poorly understood and difficult to optimize in comparison to hybridization interactions in liquid phase. For example, inconsistencies can be introduced during the creation of DNA samples that are deposited or synthesized on the solid matrix. Furthermore, diffusion parameters and the limited accessibility of DNA fixed onto the chip to test samples all conspire to make quantitation and reproducibility difficult. Second, microarrays require a relatively large amount of input RNA to achieve high sensitivity, particularly when rare genes are assessed. Third, microarrays have a limited dynamic range. Cells express RNA significantly over four or five orders of magnitude, and microarrays are only capable of working within one or two orders of magnitude. These issues of input

material severely constrain their applications in biology where the investigator wants to assay the transcriptome in small groups of cells or individual cells. This arises often in developmental biology, neurobiology and increasingly in other biological fields. Arrays typically require material from 10^6 cells or more per 10,000 genes measured. Fourth, microarrays are technically difficult to fabricate, and have a high per-experiment cost. Finally, quantitatively accuracy is limited since microarrays meant to deliver identical results differ by greater than 200%, which is greater than biologically significant differences in gene expression. Sensitive computer algorithms used to evaluate microarray data do not rectify this problem since they perform poorly with high or variable noise levels in the data.

There is thus a need for a highly sensitive method for detecting nucleic acids in biological samples. The present invention meets that need and more.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides a method of detecting a specific nucleic acid in a sample. The method includes contacting the nucleic acid with a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag, in a reaction mixture under conditions that allow the first and second oligonucleotides to specifically hybridize with the nucleic acid. The first and second oligonucleotides hybridized in such a way that the first oligonucleotide is located immediately adjacent to the second oligonucleotide to form adjacently hybridized first and second oligonucleotides. Next, the adjacently hybridized first and second oligonucleotides are ligated to form a ligated oligonucleotide, and the detector tag associated with the ligated oligonucleotide is identified.

Another embodiment of the invention provides a method of detecting a plurality of specific nucleic acids in a sample. The method includes contacting each specific nucleic acid with an oligonucleotide pair in a reaction mixture under conditions that allow the oligonucleotide pair to specifically hybridize to the nucleic acid such that the oligonucleotide pair members are located immediately adjacent to each other thereby forming an adjacently hybridized oligonucleotide pair. Each oligonucleotide pair comprises a first oligonucleotide linked to a selector tag and a

second oligonucleotide linked to a detector tag. Each adjacently hybridized oligonucleotide pair is ligated to form one or more ligated oligonucleotides; and the one or more detector tags associated with the one or more ligated oligonucleotides is identified.

Still another embodiment of the invention provides a method of detecting a nucleic acid in a sample. The method includes amplifying the nucleic acid with a primer pair to form a dual-tagged amplification product in a reaction mixture. The primer pair is a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag. Following amplification, the detector tag associated with the dual-tagged amplification product is identified.

Yet another embodiment of the invention provides a method of detecting a nucleic acid in a sample. The method includes contacting the nucleic acid with an oligonucleotide linked to a detector tag under conditions that allow the oligonucleotide to specifically hybridize to the nucleic acid to form a mixture of hybridized oligonucleotide and unhybridized oligonucleotide. A next step includes separating the hybridized oligonucleotide from the unhybridized oligonucleotide; and identifying the detector tag, thereby detecting the nucleic acid.

Another embodiment of the invention provides a kit containing an oligonucleotide primer pair and an agent that binds to the selector tag. The primer pair includes a first selector oligonucleotide linked to a selector tag and a second selector oligonucleotide linked to a detector tag.

Still another embodiment of the invention provides a kit containing a first selector oligonucleotide linked to a selector tag, a second selector oligonucleotide linked to a detector tag, and a DNA ligase.

Another embodiment of the invention provides libraries of oligonucleotides. The oligonucleotides can be linked to detector tags and selector tags.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the design of three different detector oligonucleotides (SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5) shown annealed with two corresponding target RNAs (SEQ ID NO:2 and SEQ ID NO:4). In use, the linker (CL) joining the oligonucleotide to the detector tag would be cleaved after those oligonucleotides that have hybridized with target RNA (or DNA) are separated from detector oligonucleotides that have not annealed (*e.g.*, tag 3). The liberated tags (1 and 2) would then be mixed with isotopic internal standards and detected by MS. The squares and triangles, in the peptoid implementation, represent peptoid monomers of different molecular weights, combined in a library having a known distribution.

Figure 2 shows the schematic for use of mass tagged detector oligonucleotides. Detector oligonucleotides are as shown in Fig. 1. An alternative separation scheme would first remove all single stranded DNA, thus removing unhybridized material before the poly A or other enrichment for hybridized material.

Figure 3 shows the use of selector oligonucleotides and ligation to increase specificity of mass tagged expression detection and to improve signal to noise ratios. Example uses standard oligonucleotide ligation and biotin/avidin separation.

Figure 4 shows peptoids or N-substituted glycines and their relationship to peptides. Oligomers of length two to approximately forty residues can be formed. The four structures at the bottom of the figure are exemplary peptoid residues.

Figure 5 shows peptoid synthesis via the method of Figliozzi et al. (1996). Variable R or R' represent the variable side chains chosen from among the over one thousand commercially available amines. The first step is an acylation, followed by the second step, a nucleophilic substitution. These steps are alternated until the desired chain is obtained, at which point any groups that require it are deprotected and the oligomer is cleaved from the resin.

Figure 6 shows the scheme for synthesizing a rational library of peptoids for use as detector mass tags. An interactive computer program can perform these tasks and assist in designing the most useful libraries.

Figure 7 shows the number of possible peptoids of unique mass, L, barring coincidences of different structure yet identical mass. M = length of polypeptoid and N = number of monomers of unique mass.

Figure 8 A and B shows cumulative combinations of peptoids. The variables are the same as in Figure 7, but for a given M, all peptoids that are shorter than M are also included.

Figure 9 shows a variety of strategies for linking the oligomeric mass tag to the oligomeric deoxyribonucleic acid. Both are grown on solid supports using a variety of possible growth chemistries. Some of these have the advantage of being "one-pot," others allow pre-purification, and others prevent certain undesirable chemical incompatibilities.

Figure 10 shows three molecules synthesized on a commercial oligonucleotide synthesizer. N1 is simply dT-10, and then it is modified with a disulfide-containing chain and an amide 5' terminus, both available from Glen Research. MALDI-TOF spectra of these samples are in Figures 11a-c. The terminal amine may be used as a site to initiate peptoid synthesis.

Figure 11A shows an orthonitrobenzene moiety that can be photcleaved at the location indicated by the arrow by long wave UV light.

Figure 11B shows a phosphoramidite for automated synthesis of DNA, available from Glen Research Corp., it that makes use of the orthonitrobenzene moiety to provide a photocleavable link..

Figure 11C shows that using 3-Maleimidopropionic acid, a peptoid can be modified to have a terminal maleimido group, which is specifically reactive with thiol groups

Figure 11D shows the final steps of conjugation where the peptoid and oligonucleotide are reacted at pH 7.2 in phosphate buffer for 20 hours to form conjugates.

Figure 11E shows another conjugation scheme that involves making a N-bromoacetyl peptoid, then reacting it with a disulfide oligonucleotide in the presence of TCEP (tris (2-carboxyethyl)phosphine.

Figure 12 shows the method of peptoid-oligonucleotide conjugate synthesis using the branched phosphoramidite. This method protects the ODN and the linker from peptoid synthesis chemistry.

Figure 13 shows N-Succinimidyl 3-(2-pyridyldithio) proprionate (SPDP) hetero-bifunctional crosslinker (Pierce Chemical) for condensing solution phase oligonucleotides and peptoids with a cleavable, disulfide bond. Literature reports low amounts of undesired cross-products. This scheme could be effected with either the peptoid or oligonucleotide still on support, for ease of separation.

Figure 14 shows Sarcosine-Proline-Sarcosine-Proline, made on a commercial peptide synthesizer using commercially available FMOC-Sarcosine. In actual implementation, an amide cap was added to the C-terminus.

Figure 15 shows the scheme for creation of thio-ether coupled photocleavable peptoid-ODN conjugate. There are a large number of suitable, commercially available maleimido reagents that could be substituted for EMCS.

Figure 16 shows that by mixing a pre-quantitated, cousin library of mass-shifted isotopic peptoids, duplex peaks appear on the spectrograph. The relative height of each peak in a duplex reflects the relative abundance of the two species. This overcomes the problem of deconvoluting complex mixture spectra. Five-fold deuteration of an aniline base is shown. C^{13} bromoacetic acid is another viable strategy.

Figure 17 shows a strategy in which the B-type tags were used as detectors for a second library of mRNA. Thus the relative abundance of each would express the relative differences in gene expression between those two cell types.

Figure 18 shows incorporating chromatography with the isotopic scheme for MS detection. This allows fewer duplexes to be read at a time, enhancing the signal-

to-noise ratio. Perfect chromatographic separation is not necessary as long as the masses of the tags are unique.

Figure 19 shows an example of the use of peptoid mass tags on a very large scale with applications for the microfabricated scheme.

Figure 20 shows the method for use of MAGE on a microfabricated basis.

Figure 21 shows four isotopically shifted peptoids that are chemically identical and each differ by two Daltons. D_2-bromoacetic acid was used for one acetylation in oligomer B, two in oligomer C and three in oligomer D.

Figure 22 shows that by using two complete sets of peptoid conjugates, one isotopically shifted from the other, two samples can be interrogated simultaneously, producing comparative data like microarrays.

Figure 23 shows a MALDI TOF spectrum of an n-bromoacetyl peptoid with doublet peaks because bromine is found in nature in two isotopes 2 AMU apart in nearly equal quantity. Thus, not only is the correct mass evidence of the species, but so are the doublet peaks. This is a pentamer of methoxyethylamine with a bromoacetyl tail.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method to detect and quantitate one or more specific nucleic acids in a sample. One major use is for the assay of the transcriptome (the set of RNAs expressed in a given sample of cells, tissue, or organisms) or portion thereof. The invention can be used to assay specific DNA sequences in chromosomal DNA or in DNA samples cloned and amplified by PCR or by other methods. The nucleic acids detected can be quantified using mass spectrometry in combination with isotopically labeled standards.

Invention methods address many of the drawbacks in currently available methods for assessing gene expression. Invention methods, for example Mass-Spectrometric Analysis of Gene Expression (MAGE) allow a parallel, unambiguous quantification of differentially expressed genes, and would be particularly suited to

quantifying those genes that expressed in low abundance. The method has a dynamic range at least as large as that of the transcriptome itself. MAGE can be performed on a minimum amount of material, as little as a single cell, resulting in reliable quantification. MAGE also provides a method for simultaneously measuring nucleic acid abundance more easily than two at a time (*i.e.,* greater-than-two-color assay). Furthermore, invention methods can be practiced with starting material from total RNA or merely cell lysate, instead of purified mRNA.

MAGE would provide data with great robustness and well understood error sources, so that the data would have the maximum amount of utility in the variety of data mining techniques that are being developed. This tool would also eliminate the need for reliance on housekeeping genes as standards, and it would be capable of providing transcript counts like SAGE, but without the expense or difficulty of sequencing. The assay should be simple to operate, based on well-understood physics, and be as rapid and low-cost as possible.

The invention provides a method of detecting a specific nucleic acid in a sample. Specific nucleic acids are typically messenger RNA (mRNA) or cDNA and therefore code for a gene of interest. In addition specific nucleic acids of other types, *i.e.,* hnRNA, rRNA, tRNA, snRNAs, and the like are also detectable by invention methods. RNA can be obtained from any organism and collected from a single cell, group of cells, tissues or organs. The transcriptome contains a wide variety of mRNA species. The majority of genes in the genome are expressed in very small quantities (Table 1). Furthermore, most of the very tightly regulated genes, which are of great interest , are in this scarce category. The total number of mRNA molecules in each class can be estimated by multiplying the estimated copies per cell of each mRNA sequence by the number of different mRNA sequences each class. Each specific nucleic acid contains at least one known sequence that serves as a target sequence.

	Copies per Cell of Each mRNA sequence		Number of Different mRNA Sequences in Each Class		Total Number of mRNA Molecules in Each Class
Abundant class	12,000	X	4	=	48,000
Intermediate class	300	X	500	=	150,000
Scarce class	15	X	11,000	=	165,000

For each target sequence that one wants to detect, one or more oligonucleotides are designed that specifically hybridizes with the target sequence. Oligonucleotides have a tag linked to it, with such tag being removable under appropriate conditions. Two classes of tags are used in the invention: detector tags and selector tags.

As used herein, a detector tag is a chemical moiety that can be detected by mass spectrometry which also provides a means of quantification. Each nucleic acid target sequence is associated with a tag having a specific mass. Peptoids are useful tags because they are compatible with nucleic acid hybridization of the oligonucleotide to which they are attached.

Peptoids, as used herein, are oligomers of N-substituted glycines (or "oligopeptoids," (see Fig. 4, and Figliozzi et al., 1996, *Methods Enzymol.* 267:437-47, incorporated by reference herein). Peptoids are well suited for generation of tags and also for creating combinatorial libraries of such tags (see *e.g.,* Linusson *et al.* (1999) Molecular Diversity 4:103-114). They have a single repeated linking chemistry scheme, a wide variety of monomer substitutions can be chosen, and they are thermally, chemically, and biologically stable. The behavior of peptoids in high-performance liquid chromatograph (HPLC) and capillary electrophoresis has been well studied (see *e.g.,* Robinson *et al.,* J. Chromatography B (1998) 707:247-255; Robinson *et al.,* Journal of Chromatography B, (1998) 705:341-350; Barron. Vreeland, Polymer Preprints (2000) 41:1018-1019; Heerma *et al.,* J. Mass Spectrometry, (1997). 32:697-704; and Wagner *et al., Combinatorial* Chemistry and High Throughput Screening, (1998) 1:143-154) Creation and manipulation of the libraries of peptoid tags via a robotic protocol are relatively easy because the peptoids are synthesized on solid phase supports and have a high yield at each step. Isotopic

tags, by used of ^{13}C-bromoacetic acid or isotopic amine residues, can easily be incorporated into peptoids. Peptoids do not naturally occur and thus "look" distinctly different from peptides and other naturally occurring components of cell lysates and other biological samples.

Synthesis of peptoids is accomplished by a two-step submonomer reaction cyle using methods known to those of skill in the art (see Figure 5). An initial acylation step is followed by nucleophilic substitution. These two steps are repeated until a peptoid chain of the desired length is reached. Substitutent groups are then deprotected (if protection from synthesis conditions was needed) and the peptoid is cleaved from the resin.

There are over one thousand candidate amines that can be purchased commercially for use in peptoids, but perhaps as few as twenty or thirty highly suitable ones can be used in invention methods. Exemplary amines that can be used in invention methods include benzylamine, methoxyethylamine, propylamine, phenethylamine, glycine, serine, aniline, butylamine, pentylamine, hexylamine, cyclohexylamine, methylcyclohexylamine, bromoaniline, choloroaniline, ethanolamine, furfurylamine, methylamine, ethylamine, 2,2-diphenethylamine, tyramine, ethylcyclohexylamine, methoxypropylamine, butylene diamine (for creating peptoid dendrimers), and the like. Essentially it is desirable that incorporation of one or more amines results in peptoids that are water soluble and do not bind nonspecifically to nucleic acids, *e.g,* DNA, or other peptoids. The amines should substitute, *i.e.,* be incorporated into the peptoid, with high yield, and the amine-containing products should be bromoacetylated in high yield. It is desirable that the amines included in the peptide have a range of masses and general hydrophobicity so that the resulting peptoids can be separated using a variety of convenient chromatographic methods. The amines should not contain any unprotected functionalities that will interfere with any other chemistry involved in invention methods.

Peptoids can be produced in small numbers using solid phase synthesis vessel methods, or in large numbers in parallel using robotic protocols much like those for

peptides and oligonucleotides. They are grown on the same solid supports used for peptides. They can be extended to longer than thirty bases if necessary, in high yield, and are cleaved from the support in a fashion that depends on the support chosen, but often acid or base labile linkers are chosen since they are easily available.

Peptoids can serve as detector tags because they are chemically compatible with the other chemistries required and they are relatively easy to make. Furthermore, the N-substitution, independently chosen on each repeating peptoid unit, allows the construction of a library of peptoids having a rationally determined distribution of one or several properties, such as mass, charge, size, shape, fluorescence, polarity, etc. The peptoids can range in mass from about 300 Da to about 5000 Da. For example, a peptoid constructed using five successive methoxyethylamine submonomer substitutions weighs 592 Da and is quite suitable.

When more than one peptoid detector tag is in a reaction mixture, *i.e.,* when more than one nucleic acid in a sample is detector or when a peptoid library is used, the peptoids should be separated by at least as much mass as each peptoid is separated from its isotopic control, *e.g.,* if peptoid and peptoid isotopic control are separated by 5 Da, then the next peptoid (for the next gene) should be another 5 or so Da heavier. Alternatively, the peptoids could be staggered to fit between each other, assuming that one can compensate for natural isotope abundances. With respect to charge, a negative or neutral charge is desirable. One or more negative charges per amine submonomer can be used. Total charge per peptoid can range from zero to a number depending on the length of the peptoid and choice of amines. When ten amines with a free carboxylic acid each (*e.g.*, glycine) are used, a -10 charge is obtained. This can be determined using a zeta potential machine. A negative charge would not attract DNA, while a positive charge would bind DNA non-specifically, interfering with the recognition event of hybridization. With respect to size of peptoids, bulky amines like diphenethylamine could restrict folding/bunching of the peptoid while small amines such as ethylamine or propylamine will reduce the peptoid's overall size and might allow more bunching. The relative bulkiness of the molecule would affect its properties in separation techniques that use size such as size exclusion

chromatography. Certain particle sizes can be large enough to visualize with scanning electron microscopy. Peptoid oligomers are a variety of three-dimension shapes. They are known to form various structures, some as elaborate as alpha-helices. The degree of hydrophobicity of a peptoid is a critical property. The peptoid will need to be almost completely water soluble. Methoxyethylamine, ethanolamine, glycine, and the like, for example, are more water soluble than benzylamine, proylamine, and the like, and peptoids formed from those amines have similar characteristics. A peptoid trimer of benzylamine submonomers is not water soluble. A peptoid pentamer of two methoxyethylamines, two benzylamines, and a glycine, is water soluble. Additionally, to aid in tracking the peptoids during the method, peptoids can be tagged with a moiety that absorbs or fluoresces, *e.g.,* fluorescein, rhodamine, bodipy™ differently from the sample, *e.g.,* a cell lysate, and other elements involved in invention methods. An example of this rational design is depicted in Figure 6. Numerical examples follow in Figures 7 and 8.

A large number of peptoids of unique masses can be generated using only a small number of primary amines of different masses (Table 2). L oligomers of unique mass (combinations of monomers, not permutations of monomers) are possible given a maximum oligomer length of M and N primary amines of different mass to use as submonomers. The analytical expression that generates the data in Table 2 is a cumulative combination expression: $L = \sum_{i=1}^{M} \left(\frac{i+N-1}{N} \right)$.

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The generative function for the examples shown does not account for species with identical masses but different structures—these species can be eliminated if the library is created combinatorially, or not created in the first place if the library is created piece-wise. The entire human genome could be encoded with nine-mers formed from only nine different primary amines. Significant clusters of genes could be studied with tetramers formed from four different primary amines. It is probably beneficial to use shorter peptoids to increase mass resolution in the detection phase and increase the oligomer-to-oligomer differences for chromatography.

Table 2

	M=1	2	3	4	5	6	7	8	9
N=1	**L=1**	2	3	4	5	6	7	8	9
2	2	5	9	14	20	27	35	44	54
3	3	9	19	34	55	83	119	164	219
4	4	14	34	69	125	209	329	494	714
5	5	20	55	125	251	461	791	1286	2001
6	6	27	83	209	461	923	1715	3002	5004
7	7	35	119	329	791	1715	3431	6434	11439
8	8	44	164	494	1286	3002	6434	12869	24309
9	9	54	219	714	2001	5004	11439	24309	48619

A library of peptoids with unique, masses distributed over a wide range, and having properties that are compatible with hybridization conditions, cleavage conditions, and enzymatic steps, *e.g.*, ligation, can be designed. The library can be catalogued so that each unique mass is associated with the oligonucleotide that it tags. Optionally, amines can be incorporated into certain peptoids that give the peptoids a strong and unique signature that can be readily observed with a detection device, to allow easy quantitation of single species, and for the purposes of generating stock solutions. For example, amines that provide peptoids with a strong signal using UV/Visible detection can be used. Peptoids are also very stable to storage on resin or as a lyophilized powder.

The manner in which the peptoid detector tag is linked to the oligonucleotide is crucial for the success of invention methods. The linker must be relatively easy to form and the chemistry used to form it must not damage the tag or the

oligonucleotide. The reaction should be highly specific and allow complete purification of the products which reduces the contribution to noise during the quantitation step. Very importantly, the linker must resist breakage during any further oligonucleotide preparation steps, the hybridization step, and any separation steps. When the linker is cleaved, it must cleave in high yield, preferably quantitatively, in a consistent manner. The chemistry used to cleave the linker must not damage the detector tag, for example, a peptoid.

Several general strategies can be to generate an oligonucleotide to a detector tag (see Figure 9 and Examples 1 to 4). Most start with a solid support such as a resin. Using chemistry known in the art, DNA residues can be attached in a sequential manner followed by attachment of a linker, followed by sequential attachment of peptoid residues. Alternatively, peptide residues can be attached in a sequential manner to the resin, followed by the linker, followed by sequential attachment of DNA residues. In another strategy, a pre-formed peptoid having one or more residues is attached to the resin, followed by attachment of DNA residues connected together, *i.e.*, an oligomer, which has a linker attached. The converse strategy, *i.e.*, attachment of a pre-formed oligomer followed by attachment of a preformed peptoid having a linker attached. When a solid resin support is not used, a pre-formed peptoid containing a linker can be attached to a pre-formed DNA residue. The strategy chosen depends on whether pre-purification of the reaction product is desired, and whether undesirable chemical incompatibilities can be avoided.

Detector oligonucleotides (when the detector tag is a peptoid) can be constructed with a variety of schemes. Since both oligonucleotides and peptoids are synthesized on solid-phase supports, one could synthesize them subsequently (or "on-line"). This requires that either the peptoid be exposed to oligonucleotide synthesis conditions, or that the oligonucleotide be exposed to peptoid synthesis conditions. This has been done successfully with peptide-oligonucleotide conjugates, but a considerable amount of adjustment has to be made to standard synthesis procedures, which is not convenient with a shared facility synthesizer (Truffert *et al.*, Tetrahedron Letters, (1994) 35:2353-2356 and de la Torre *et al.*, Tetrahedron Letters, (1994)

35:2733-2736. A further difficulty is adapting peptoid chemistry, which is based on a submonomer scheme that makes use of harsher reagents than that of peptide chemistry, to the on-line synthesis scheme. The most straightforward method for on-line synthesis is to produce the oligonucleotide of interest, add one of several phosphoramidites that contain a cleavable linker, and then terminate the nucleic acid portion of the conjugate with a 5' amine modification. This primary amine could be used to initiate a peptoid synthesis. This process has a low yield and the purine nucleic acids may not successfully withstand the direct acetylations used in peptoid synthesis. Any one-pot scheme that is attempted requires significant modification of either the peptoid synthesis conditions or the oligonucleotide synthesis conditions, and considerable optimization. It also leads to a complex final purification step.

In a two-pot scheme, the oligonucleotides and peptoids are produced via standard protocols, purified and kept for future uses. Oligonucleotide or peptoid, or both, is modified either during their solid-phase syntheses, or afterwards, and they are coupled. The final product mixture is simpler to purify than the result of the one-pot scheme because there is only one representative of each species of oligomer, instead of the normal mixture of partial products produced during solid-phase synthesis.

There are two major options for the linkage chemistry. Either the cleavable moiety is added prior to the conjugation, which is done via a permanent linkage, or the conjugation leads to the formation of a cleavable linker directly. In the latter method, the typical choice would be to form a disulfide linker between the oligomers under oxidizing conditions, which can later be reduced. The disulfide bond is not extremely stable across a variety of conditions, and the most worrisome trait of a cleavable linker would be premature cleavage, because that would result in lost signal. One method for performing a post-synthetic conjugation (Figure 13) that leads to a disulfide bond is to make use of the heterobifunctional crosslinker SPDP (N-Succinimidyl 3-(2-pyridyldithio)proprionate).

Alternatively, the cleavable moiety could be added prior to conjugation, either to the peptoid or to the oligonucleotide. A commercially available phosphoramidite,

the building block for automated DNA synthesis, contains a alkyl chain broken by a disulfide bond. Normally, this is reduced post-synthesis so it can be used as a terminal thiol group. If it were left on, a further phosphoramidite could be added 5' to the disulfide bridge that contains a free amine. The amine could be used for a permanent conjugation to a peptoid. One slightly modified version of this scheme makes use of a commercially available branched phosphoramidite (Juby *et al.*, Tetrahedron Letters (1991) 32:879-882) that has the Fmoc protecting group used for peptide synthesis and the DMTO protecting group used for oligonucleotide synthesis (Figure 12).

Another method for generating a cleavable linker is to make use of recently developed bio-compatible photolabile moieties based on ortho-nitrobenzene (Figure 11A). No mechanism has been indicated yet, but if a polymer chain either passes through the phenyl ring, or is adjacent to the phenyl ring, and the orthonitro group is neighbor to another bond such as an amide or phosophodiester, then long-wave ultraviolet light can cause the neighboring bond to cleave. The extent of cleavage and the products depend on the configuration of the neighboring groups. Linkers based on ortho-nitrobenzene are used often for solid-phase supports, when the chemistry to be done on the solid phase involves both acidic and basic conditions, so a linker is required that is stable to both acid and base.

The most efficiently cleaving implementation of the ortho-nitrobenzene moiety was developed recently for use in DNA oligonucleotides (Olejnik *et al.*, (1996) Nucleic Acids Research 24:361-366). Optimization of neighboring groups and structures yielded a cleavable linker that is stable to acid and base, and cleaves quantitatively in five minutes upon exposure to long-wave ultraviolet light Olejnik *et al.*, (1999) Nucleic Acids Research 27:4626-4631). This linker has been incorporated into phosphoramidites and is now available commercially. One disadvantage of this linker is that oligonucleotides containing it are difficult to characterize. MALDI is often used for mass spectrometry of oligonucleotides, and since most MALDI systems ionize the sample with an ultraviolet laser, the sample is partially cleaved in the process. This property is used for solid-phase assays involving this linker (Hahner *et*

al., (1999) Biomolecular Engineering 16:127-133). The most appropriate version of this phosphoramidite is commercially available in a photocleavable-spacer version (Figure 11B); A phosphoramidite for automated synthesis of DNA, available from Glen Research Corp., it that makes use of the orthonitrobenzene moiety to provide a photocleavable link.

Once the cleavable linker is incorporated into the oligonucleotide during the automated synthesis, it is followed by a 5' modifier that will allow the oligonucleotide to be bound to the peptoid. One possibility is to use a 5' amino modifier and continue the synthesis of the peptoid on the same resin. This suffers from the disadvantages of on-line synthesis as discussed herein. Another method would be to use the branched phosphoramidite scheme (Fig. 12) with the photocleavable-spacer instead of the disulfide bridge. This would allow the peptoid to be bound 3' to the oligonucleotide instead of 5'. A common method for post-synthetic conjugation yielding a peptoid 5' to the oligonucleotide is to condense solution phase oligomers to form a thioether bond. The oligonucleotide is synthesized with the cleavable linker, and then terminated with a 5' thiol modification. The peptoid is synthesized separately, and after the final primary amine is substituted, an extra acetylation step is performed using 3-maleimidopropionic acid (Figure 11C). In the final steps, the peptoid and oligonucleotide are reacted at pH 7.2 in phosphate buffer for 20 hours to form conjugates (Figure 11D). Another conjugation scheme involves making a N-bromoacetyl peptoid, then reacting it with a disulfide oligonucleotide in the presence of TCEP (tris (2-carboxyethyl)phosphine (Figure 11E).

Removing the detector tag is performed by subjecting the a moiety having a linked detector tag, for example, a ligated oligonucleotide according to invention methods, to a de-linking agent. A de-linking agent, as used herein, refers to the agent used to cleave the linker that attaches a detector tag to a nucleic acid. The mechanism of the agent, for example, the chemical conditions, must be orthogonal to the other chemical reactions used in the assay. De-linking agents include an acid condition, an alkaline condition, visible light radiation, UV radiation, heat, a reducing condition and an oxidizing condition.

Invention methods include the step of contacting the nucleic acid of interest with at least one oligonucleotide. An oligonucleotide is linked to a selector tag or to a detector tag. Contacting is performed under conditions that allow the oligonucleotides to specifically hybridize with the nucleic acid. Typically, hybridization of mass-tagged detector oligonucleotides with the target nucleic acid population is done under conditions of molar excess of oligonucleotide detectors to target so that little or no desired target remains unhybridized, and under kinetic conditions such that the hybridization is stopped near or beyond kinetic termination. The reaction conditions are also chosen with attention to hybridization stringency so as to favor stable interaction of detector oligonucleotides with their intended target sequences, while at the same time being as unfavorable as is possible for interactions of the detector with non-target RNAs.

As used herein, "specific hybridization" refers to hybridization under low stringency, moderately stringent or highly stringent conditions which distinguishes related from unrelated nucleotide sequences. (See, for example, the techniques described in Maniatis *et al.*, 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference),

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether target nucleic acids are contacted in solution or rather than immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be

used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the

Following a hybridization between one or more detector oligonucleotides and a preparation containing potential target nucleic acids, those mass-tagged primers that have hybridized will be separated from the primers that have not hybridized with target nucleic acids. Un-hybridized oligonucleotides can be present because all legitimate targets, that is the nucleic acids of interest, are already hybridized, or because there were no target nucleic acids, *i.e.*, RNAs, complementary to a detector oligonucleotide species. In certain embodiments of the invention, separation is not necessary. In other embodiments, separation can be accomplished by several possible means.

Physical separation of the hybridized detector oligonucleotides from non-hybridized ones (and from other molecules in the reaction mixture) may be accomplished by any of several strategies. These strategies can be designed to be more or less rigorous for eliminating non-hybridized material depending on the signal to noise ratio required during identification of the detector tag. For small input samples, more rigorous separation strategies are usually employed, with a reduction for samples where larger input material is readily available. Contamination with other non-tag material is a second consideration in separation strategies. In a simple form, if the peptoid library is rationally designed, non-expected masses can be discarded. The peptoids that serve as detector tags can also be modified to contain a variety of useful chemical "hooks" or "probes" that would further facilitate easy separation, such as fluorescence, charge, biotin, and the like.

To select hybridized over non-hybridized detector oligonucleotides when the detector oligonucleotide is hybridized to mRNA, one separation method uses the fact that virtually all mRNAs have a poly A tail at their 3' end. Methods for purification on the basis of the presence of poly A are well established. Oligonucleotides having peptoid detector tags are well-suited to this type of separation because the peptoids do not interfere with the separation. Similarly, the presence of 5' cap structures on the

RNA can be employed. Using the poly A separation scheme, all mRNAs and detector oligonucleotides that are stably associated with mRNA, would be separated from unhybridized oligonucleotide. The power of the separation can be enhanced (with a resulting increase in purification and downstream increase in signal to noise ratios) by either combining this with another selection method or by performing multiple iterations of this selection.

High-performance liquid chromatography or capillary electrophoresis are both capable of purifying the probe-target duplexes. The addition of other selector moieties can aid in selecting desired hybridization or amplification products, For example, a distribution of fluorine atoms could be incorporated on the peptoids' amine submonomers, and a fluorous HPLC column could be used to retain the peptoid tags while unmodified nucleic acids pass through.

Another separation strategy takes advantage of the selector tag linked to oligonucleotides, *i.e.,* selector oligonucleotides. Various selector oligonucleotides and separation (purification) methods associated with the various selector oligonucleotides can be used in invention methods. Conjugation of a selector oligonucleotide with a physical or molecular tag that can be used to separate the oligonucleotide allows the separation of unhybridized oligonucleotides linked to selector tag and hybridized oligonucleotide linked to selector tag, for example selector oligonucleotide hybridized to target nucleic acids. The selector tag can be used to separate the selector oligonucleotide from a reaction mixture using an agent that binds to the selector tag. For example, a biotinylated selector oligonucleotide can be separated using avidin/strepavidin binding methods. A selector oligonucleotide tagged with digoxygenin can be separated using anti-digoxygenin affinity reagents. An oligonucleotide linked to a magnetic selector tag, for example, paramagnetic beads, can be separated using a magnetic field. Selector tags can be enzymes with selection by enzyme activity (or the converse). The selector tag can be a fluorescent dye or dye impregnated bead that would allow the use of optical sorting, or would serve as a detection device during or following chromatographic separation. The selector tag can have a very prominent chromatographic feature such as charge or size

that would allow simple purification using an ion-exchange column or other column. Exemplary selector tags include tags: a fluorescent compound, a luminescent compound, a chemiluminescent compound, a radionuclide, a paramagnetic compound, and biotin.

In some embodiments of the invention, selector tags are the same for all selection primers when a plurality of selector oligonucleotides is used, rather than specific for each target as the detector tags are. In another embodiment, more than one family of selector primer might be used in the same hybridization mix. This would act as an internal standard within each experiment or to generate more than one family of products for later detector tag analysis, *i.e.,* multiplexing. For example, in a simple form, two selector tags can be used with each selector tag used in combination with the same group of detector tags to detect the same group of targets. The hybridized target nucleic acids would be separated from the reaction mixture and into two groups based on the selector tag, and processed independently (removal of the detector tags). Since the selector tag does not interfere with hybridization, each selector tag-selected nucleic acid group should be the same. The two-fold redundancy provides and internal control for method reliability.

In an alternative embodiment, a primer family can be used to increase the multiplexing capacity of the method. For example, when identification by mass spectrophotometric analysis is limited to simultaneously detecting about 20 to30 peptoids, a family of selector tags, from about 5 to about 10, about 15, or about 20, can be used in combination with a set of detector probes. In other words, the same detector tag can be associated with more than one oligonucleotide probe or primer and each detector tagged oligonucleotide that hybridizes with a unique target polypeptide target sequence will be "binned" with one selector tag from a family of selector tags. This strategy can increase the total multiplexing capability in a reaction mixture by one to two orders of magnitude.

Any affinity scheme that would allow physical isolation of selector oligonucleotides from non-hybridized detector primers as classes of molecules could be used, but the enhancement of specificity is retained only if they are ultimately co-

purified due to the fact that a selector primer has become attached to a detector primer because both have annealed to the same RNA or DNA target. The type of selector tag employed depends on cost, yield, ease of manipulation, efficiency and ability to obtain good separations on varied sample quantities. In some applications of invention methods, very small amounts of sample are contemplated.

For both selector and detector classes of oligonucleotide, the particular nucleotide sequences used and the length of those sequences can be optimized for each invention method and for each particular method use. A typical length for oligonucleotides (RNA or cDNA) linked to detectors or selectors ranges from about five to forty nucleotides, but some considerations might make either shorter or longer ones appropriate. In any case, sets of detectors and selectors matched for similar Tm (a measure of melting temperature) when they are to be reacted together in the same mixture (multiplexed) is desirable. Short oligonucleotide sequences are likely to anneal to multiple genes. Short oligonucleotides (*e.g.*, 5 to10 mers) can be used in order to detect the total quantity of the complementary sequence(s) in the sample, for example, to identify a specific nucleotide motif. A motif may identify a gene family, or one gene function, but may not necessarily identify the gene itself. Long oligonucleotides (about 50 to 70-mers or as long as 100 mers) can be used to ensure as much specificity as possible. This would be helpful when the sample contains a very complex mixture of nucleic acids or when detecting a sequence in a sample that has two or more homologous sequences. When invention methods are used to identify gene expression in a sample, the oligonucleotide length chosen for the primer or probe is as short as necessary to specifically identify each gene

Testing of sequence sets to exclude undesirable cross hybridization with each other (both informatic tests and bench tests) can be used to optimize multiplex determinations. It is envisioned that a set of detector oligonucleotides and selector oligonucleotides would eventually be designed to be compatible with each other and to include all genes for a given organism or all gene products. Subsets could then be used at will, as the experimental or clinical case demands. Modified oligonucleotides with alterations in bases, sugar, base modifications or backbone modifications might

become desirable to enhance stability or to facilitate later separation from the tags or to improve their shelf-lives.

Following separation using selector tag, the detector tag is removed from the hybridization product. The hybridization product refers to the nucleic acid of interest to which is hybridized an oligonucleotide linked to the detector tag, and, optionally, in certain embodiments, an oligonucleotide linked to a selector tag. Strategies for removal of the detector tag are identified herein. Following its removal from the hybridization product, the detector tag is identified, allowing detection, and optionally, quantification of the nucleic acid of interest.

Invention methods exploit the advantages of mass spectrometry for nucleic acid detection and the measurement of nucleic acid abundance. This is not as straightforward as merely submitting an unknown mixture of nucleic acids to mass spectrometric analysis, because the mass of a transcript alone does not determine its sequence uniquely. Furthermore, the signal strength of an individual component in a mixture does not correspond to its abundance in the mixture since different compounds ionize to different extents in ESI (electrospray ionization) or MALDI (matrix-assisted laser desorption ionization) spectrometry. Mass spectrometry methods can be used to identify oligonucleotides in a mixture (Pomerantz *et al.*, J. Am. Chem. Soc., 119:3861-3867) but for the reasons described above, mass spectrometry can not be directly used to measure the oligonucleotides. An assay that makes use of mass spectrometry for quantification is desirable, however, because mass spectrometer sensitivity has reached the zeptomole region (about 6000 molecules) and below, and dynamic ranges in excess of six orders of magnitude. This level of sensitivity exceeds any available method for reliable single-transcript quantification, much less any multiplexed method, and is invaluable when assessing of scarce transcripts. Thus, an indirect method of quantification using mass spectrometric analysis is used in invention methods.

The method of isotopic internal standards will be used to quantify the tags. A scheme based on isotopic labeling can ameliorate complications of quantification due to variations in ionization and other effects (see Figures 16 and 17). Each peptoid

mass tag will have a chemically identical "cousin" that has been isotopically labeled to be a fixed amount heavier. For example, three carbons of the backbone might be ^{13}C, or the aromatic ring of a benzylamine side chain could be five-fold deuterated. The particular amount of the shift will depend on the type of mass-spectroscopy and the presence of interfering natural isotopes.

Mass Spectrometric analysis provides high sensitivity and dynamic range (Poulsen et al.,. Rapid Communications in Mass Spectrometry (2000) 14:44-48; Walk et al., *ESI Fourier Transfer Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR-MS): A Rapid High-Resolution Analytical Method for Combinatorial Compound Libraries.* Angew. Chem. Int. Ed., 1999. 38: 1763-1765). The application of mass spectrometry, especially ESI-FTICR (Fourier Transform Ion Cyclotron Resonance) to deconvolution of combinatorial mixtures is a rapidly growing field (Fang et al., Combinatorial Chemistry and High Throughput Screening, (1998) 1:23-33; and Nawrocki et al., Rapid Communications in Mass Spectrometry, (1996) 10:1860-1864; Tutko et al., Rapid Communications in Mass Spectrometry (1998) 12:335-338). ESI is a particularly convenient ionization method, since it is used with chromatographs and is high throughput when compared to MALDI (see Wu and Odom, Analytical Chemistry, (1998): p. 456A-461A). If MALDI is necessary to resolve the peptoids, it will make data interpretation easier if the peptoid masses are not too crowded on the spectra.

With the right equipment, zeptomole detection is possible, and the dynamic range is beyond six orders-of-magnitude and tunable. For genes that are known to express in very small amounts, small amounts of isotopic standard can be added, so the peaks will be comparable. Similarly, for highly expressed genes, large amounts of standard can be added. Using the cousin peptoids as internal standards could produce exact inventory data on unknown nucleic acid concentrations, much like the SAGE method. However, to eliminate the potential need for carefully standardizing a library of cousins, the entire MAGE assay could be run on two samples (Figure 22). In the case of gene expression, cells in two different states could be used. One sample would be exposed to the regular peptoid library, the other, to the modified peptoid

library. After the peptoid tags are purified, the two resulting mixtures are cotransduced in the mass spectrometer. The relative peak sizes then are a measure of the relative gene expression in the two samples, producing data much like that of DNA microarrays.

Oligonucleotide or nucleic acid sequence refers to a polymeric form of nucleotides. The terms include, for example, a recombinant DNA or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term polynucleotide(s) generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, oligonucleotides, as used herein, refers to, among others, single-and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions.

In addition, the oligonucleotides or nucleic acid sequences may contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "oligonucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

In embodiments of the invention, when a library of peptoids is used as detectors, a second, shifted library is constructed in a known concentration. This library is mixed with the hybridized and purified tags, and serves as an internal standard. With no chromatographic separation, the resulting spectrum will have a large number of peaks, each with a cousin a fixed distance away. The key matter, then, is that because both tags are chemically similar, they will ionize to the same extent. Thus, relative to the internal standard, the amount of the unknown tag can be assayed by comparing the peaks.

Additionally, a chromatographic step, such as LC-MS or CE-MS, can be added. This will produce the data one or several peaks at a time, and should increase the signal to noise ratio. Again, because the cousin tags are chemically identical, they will always elute at the same time, and show up on the spectrum as a duplex (Fig. 21).

Another method that might be used is to have a method of cleaving detector from selector tags, and add an isotopic standard selector tag. This could be used for assaying the total amount of mRNA hybridized as a control.

One key area that the use of MS addresses is sensitivity and dynamic range. With the right equipment, single-molecule detection is possible. And the dynamic range is also very great, and tunable. For genes that are known to express in very small amounts, small amounts of isotopic standard are added, so the peaks will be comparable. Similarly, for highly expressed genes, large amounts of standard are added.

The detached tags are analyzed by mass spectrometry, using an internal standards method described later. At the end of this process, the user will deduce whether a particular RNA sequence was present in the original sample (cell lysate, RNA preparation, or other biological sample containing RNA or a nucleotide representation of the RNA such as cDNA) by the presence of the corresponding mass signal from the detector primer (or group of detector primers) that were specific for that mass signal from the detector primer (or group of detector primers) that were specific for that RNA. The presence and absolute or relative amount of a given mass tag will reflect the amount of the target complementary RNA present in the original sample. One can also detect or quantitate sequences corresponding to different parts of a single RNA or cDNA polynucleotide. In general, multiple different oligonucleotides directed at different parts of the same gene will be used as informative internal controls, and (if the same mass tag is used for multiple oligonucleotides directed at the same target) this can also be used to raise sensitivity for a rare target RNA. Other variations on these latter manipulations might also be used in special biological instances to measure the relative concentrations of intron

versus exon sequence for a single gene in the sample of the level of 5' end versus more 3' sequences for a particular gene's transcript population.

In another embodiment of the invention, a method is provided for detecting a specific nucleic acid in a sample. The method includes contacting the nucleic acid with a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag under conditions that allow the first and second oligonucleotides to specifically hybridize with the nucleic acid such that the first oligonucleotide is located immediately adjacent to the second oligonucleotide, thereby forming adjacently hybridized first and second oligonucleotides.

In this method, the target is cDNA made from the RNA in the sample and the detector primers are DNA or any variation on DNA that can be used by DNA ligase as a substrate. Following hybridization to target cDNA with detector primers and with selector primers, a reaction is performed on the mixture using DNA ligase as the catalyst. Such ligation reactions result in formation of a covalent phosphodiester link between the 3'-most residue of one oligonucleotide and the 5'-most residue of the adjacent annealed oligonucleotide or polynucleotide. The absolute and highly precise requirement for the placement of detector and selector oligonucleotides on the same target cDNA is extremely powerful for improving specificity. For example, if in a single hybridization method, the specificity = X, using this method should improve specificity to a value greater than X squared. Thus the selector and detector oligonucleotides must be precisely adjacent relative to each other and the nucleotides that are joined by ligase must be correctly base paired with the target cDNA. Other embodiments of the invention employ other oligonucleotide species, *e.g.*, RNA, various synthetic analogs, and corresponding ligases, *e.g.*, RNA ligase.

The contacting is carried out in a reaction mixture. Following hybridization, in the reaction mixture, there are the adjacently hybridized first and second oligonucleotides, that is a nucleic acid with the first oligonucleotide specifically hybridized to it and the second oligonucleotide specifically hybridized to it so that the first and second oligonucleotides are immediately adjacently hybridized. Also following hybridization, there are unhybridized first oligonucleotide linked to selector

tag, unhybridized second oligonucleotide linked to detector tag, first oligonucleotide hybridized to a nucleic acid without hybridization of second oligonucleotide, and second oligonucleotide hybridized to a nucleic acid without hybridization of first oligonucleotide.

As used herein, "immediately adjacently hybridized" refers to two nucleotides that are complementary to neighboring sites on a nucleic acid so that following hybridization, the 5'-P and the 3'-OH termini of the oligonucleotides can be ligated by the formation of a 5'-3' phosphodiester bond. Ligases such as DNA ligase, for example T4 DNA ligase, and RNA ligase, catalyze the formation of the phosphodiester bond.

Ligation of first and second oligonucleotides forms a ligated oligonucleotide. The ligated oligonucleotide is annealed to the nucleic acid of interest. Such a ligated oligonucleotide bears two tags: one selector tag which was linked to the first oligonucleotide, and one detector tag which was linked to the second oligonucleotide. It is understood that hybridization of the oligonucleotides and ligation of the oligonucleotides does not interfere with the identification of the oligonucleotide.

Identifying the detector tag associated with the ligated oligonucleotide detects the specific nucleic acid in the sample. Identifying the detector tag comprises separating, using the separator tag, the ligated oligonucleotide from the reaction mixture. It is recognized that the selector tag, used as a means to separate the ligated oligonucleotide from the reaction mixture, will also separate from the reaction mixture, un-hybridized oligonucleotide linked to selector tag and non-adjacently hybridized oligonucleotide linked to selector tag. Un hybridized oligonucleotide linked to detector tag and non-adjacently hybridized oligonucleotide linked to detector tag (and remaining components of sample) remain in the reaction mixture.

Using the selector tag to separate the ligated oligonucleotide from the reaction mixture can be accomplished as described herein. Briefly, the selector tag can be contacted with an agent that specifically binds to the selector tag. Identifying the detector tag can be accomplished as described herein.

In another embodiment of the invention there is provided a method for detecting a plurality of specific nucleic acids in a sample. The method comprises contact each specific nucleic acid with an oligonucleotide pair under conditions that allow the oligonucleotide pair to specifically hybridize to the nucleic acid so that the pair members are located immediately adjacent to each other. Each oligonucleotide pair contains an oligonucleotide linked to a selector tag and an oligonucleotide linked to a detector tag. The method includes ligating each adjacently hybridized oligonucleotide to form one more ligated oligonucleotides and identifying the one or more detector tags associated with the ligated oligonucleotides.

A plurality of oligonucleotide pairs is used for detecting a plurality of nucleic acids in a sample. Each oligonucleotide pair contains a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag. Libraries of oligonucleotides are provided by invention methods. One such library contains a plurality of first oligonucleotides linked to selector tag. Each oligonucleotide is complementary to a specific nucleic acid or specific nucleic acid target sequence. Such a library can be used to identify a variety of genes, for example to assess gene expression in a sample. The selector tag on each oligonucleotide is not different for each nucleic acid target. The selector tag is chosen because it provides a facile handle for retrieving and physically separating properly hybridized detector oligos through their physical linkage to a companion selector primer. Another library contains a plurality of second oligonucleotides linked to detector tags. Each oligonucleotide is complementary to a specific nucleic acid or specific nucleic acid target sequence. The detector tag on each oligonucleotide is different for each nucleic acid target. A unique detector tag on each detector oligonucleotide allows the identification of the target nucleic acid in a sample. Each library described, *i.e.*, the library of selector oligonucleotides and the library of detector oligonucleotides can be combined to form a library of oligonucleotides in which a selector oligonucleotide can adjacently hybridized to a detector oligonucleotide.

Gains in specificity and in sensitivity come from joining detector and selector oligonucleotides via the polymerase chain reaction. In this embodiment, both detector

and selector oligonucleotides act as primers for DNA synthesis, with the product being a DNA duplex containing selector tag on one strand and detector tag on the other, *i.e.*, a "dual-tagged oligonucleotide duplex. The gain in specificity is well understood in the current state of the art to come from the necessity of two simultaneous, high-specificity annealing events that require the sequence content of the target RNA or DNA and its deduced complementary strand.

Accordingly, in another embodiment of the invention, there is provided a method of detecting a nucleic acid in a sample comprising amplifying the nucleic acid with a primer pair to form a dual-tagged amplification product in a reaction mixture, and identifying the detector tag associated with the dual-tagged amplification product. The primer pair is a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag.

This embodiment of the invention can significantly increase the sensitivity of the assay by increasing the number of detector tags identified with a single target sequence. Detector and selector tags are both made sufficiently stable to cycling conditions such as thermocycling. Such cycling permits specific amplification of the number of detector tags that will eventually be measured to correspond to a given target sequence. For very small samples or for rare RNA targets, this improves the certainty of detection and can favorably shift the threshold of detection limits.

The invention also contemplates a microfabricated "Lab-on-a-Chip" method. Ideally, gene expression assays would be very fast, and suited for large numbers of samples, as well as accurate. High throughput gene expression assays could be used to study cells in rapid flux, so that better time-dependent maps of gene expression can be constructed. They would also be convenient for diagnostic purposes, allowing medical staff to run these assays without complicated, expensive lab procedures. For instance, gene expression assays are currently being used to diagnose disease, in particular cancer. A rapid diagnostic tool would give physicians the ability to treat conditions with maximal effectiveness.

Because they involve either sequencing, or hybridization to solid surfaces and subsequent scanning, current methods are not amenable to high-throughput use. In contrast, liquid-chromatography mass-spectrometry (LC-MS) is rapidly becoming an important analytical technique for high throughput combinatorial chemistry, and would be well suited for the analytical portion of mass analysis of gene expression (MAGE). In one manifestation of MAGE, no solid-affinity methods are used, and the entire method could be micronized onto the surface of a chip (see Figure 20). Wells, pumps, mixing chambers, heating ovens, photo-chambers, CE, and electrospray MS have all been implemented on a chip basis in separate technologies and are known in the art. Designing the peptoid library to allow us to accomplish our separations effectively using CE would eliminate solid-affinity steps. The internal standards are mixed on the chip, and the photo-cleavage is accomplished by shining a laser of the required wavelength onto the chip. The chip would then output the material to the electrospray apparatus of a mass spectrometer. In this example, a complete gene expression assay could be accomplished from isolated mRNA, or more ideally from a cell lysate, in a very short period of time, and the same device could be reused any number of times. The wells that store the mass tags could be programmed to release in a serial fashion, thus allowing the system to multiplex at high speed. In this way, the number of genes that can be assayed could range from one to any number, even as many as the entire human genome contains.

Invention methods can also be used for rapid, large-scale genotyping by constructing a probe conjugate complementary to one version of each single-nucleotide polymorphism (SNP) being studied. The isotopic cousin peptoid would be conjugated to the probe complementary to the other version of each SNP. The ESTs or even genomic DNA (if PNA probes are used) targets are exposed to the probes, and using the protocols described herein, the relative abundance of the polymorphism can be determined. A sample from a single organism should have only one of the two peaks in the doublet, if hybridization conditions are stringent enough, but the doublet that is much larger than the other is likely to be the version of the SNP that is actually present in the sample DNA.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are to be considered illustrative and thus are not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLE 1

Exemplary oligonucleotides linked to detectors are provided. In a first example, the oligonucleotide is grown on a solid support in a conventional synthesizing robot. Then, the 5' end of the oligonucleotide is modified with two commercially available reagents, which add a disulfide and a free amine. A free amine is the usual starting place for synthesizing a peptoid on an amide resin, so from this point, the resin is transferred to a solid phase synthesis vessel and the peptoid chemistry is executed. Some success was achieved in low yield for a single peptoid base. The result of this kind of linkage is a disulfide, which is easily reduced with DTT. One potential problem is that disulfide bonds may or may not survive the peptoid synthesis and further steps.

In a second example, the peptoid is synthesized first. This avoids exposure of the oligonucleotide to the chemistry of peptoid synthesis. The scheme is depicted in Figure 10, and it relies on a commercially available phosphoramidite that has a moiety for growing an oligonucleotide as well as an FMOC group for protecting a site for initiating growth of the peptoid. This branched unit could also be combined with the photolabile phosphoramidite.

In a third example, a complete oligonucleotide and peptoid are first prepared. The peptoid is then reacted with a commercially available protein cross-linking reagent such as SPDP. This scheme is depicted in Figure 13. An example of a peptoid that could be used for the condensation reaction is "SarProSarPro," depicted in Figures 14. The end result of this process is the direct condensation of a disulfide. The difficulty of this method is that all of the incomplete products must be purified, using RP-HPLC, or possibly ion-exchange.

In this fourth, preferred example, a phosphoramidite with a photocleavable moiety built in gives a linking function with no additional chemistry. It could be combined with any number of non-cleavable linking schemes between oligonucleotide and peptoid. Many precedents have been established for successfully conjugating peptides to oligonucleotides. One preferred example is to terminate the oligonucleotide in a free thiol, and conjugate the peptoid with a maleimido group. The resulting reaction is extremely efficient and results in a stable thio-ether bond.

EXAMPLE 2

Maleimido-modified Peptoid Synthesis

Peptoid trimers, tetramers and pentamers have been synthesized in high yields manually, using the method of Figliozzi *et al,* (*supra*, see also, Table 3*)*. The synthesis described here uses 100 mg of resin, but up to 250 mg have been successfully used in the same size synthesis vessel by scaling up all other reagents linearly. 100 mg of rink amide MBHA resin (Novabiochem, La Jolla, CA) is loaded into a 10 mL peptide synthesis vessel that has been modified to improve agitation by adding a small pocket on the wall of the reaction chamber. The resin is first washed several times with N,N-dimethylformamide (DMF, Aldrich Chemical Co., Milwaukee, WI). All solvents are purchased anhydrous and kept as dry as possible by careful handling and storage with molecular sieves (3A, EM Industries, Gibbstown, NJ). The resin is agitated by an upward directed flow of Argon. A wash step refers to adding 1-2 mL of solvent, agitating for 30 seconds, then draining the vessel under aspirator vacuum.

After the initial washing to swell the resin, the DMF is drained and the Fmoc group protecting the resin's free amine is removed by adding 2 mL of 20% piperidine in DMF, agitating for one minute, draining, and adding another 2 mL of 20% piperidine. The second solution is agitated for 15 minutes and then drained. The resin is washed with DMF six times before peptoid synthesis.

At this point, repeated rounds of a single linking chemistry are used. First, the free amine is acetylated by adding 850 μL of 0.6M bromoacetic acid (BAA, Aldrich Chemical Co., Milwaukee, WI) and 200 μL of 3.2M diisopropylcarbodiimide (DIC,

Aldrich Chemical Co., Milwaukee, WI). The slurry is agitated for 30 minutes, drained, and an identical solution is added for a further 30 minutes of agitation. Following this, the mixture is drained, washed twice with DMF, and once with N-methylpyrrolidone (NMP, Aldrich Chemical Co., Milwaukee, WI).

The nucleophilic substitution of a primary amine is the second half of a round of synthesis. The primary amine of choice is dissolved at around 1.5 M in NMP and 1 mL of this solution is added to the vessel. The mixture is agitated for two hours, drained, and the resin is subsequently washed twice with NMP and once with DMF. The cycle can be repeated up to 75 times depending on the choice of primary amines. The main source of poor yield is incomplete acetylation due to excess water in the DMF mixtures.

TABLE 3

Step		Reagent	Reaction Time	Volume (μL)	Repetitions
1	BAA Addition	0.6M bromoacetic acid in DMF	-	850	-
2	Activation	3.2M diisopropylcarbodiimide in DMF	-	200	-
3	Acetylation		30 min		2
4	Wash	DMF NMP	30 s 30 s	2000 2000	2 1
5	Displacement	1.5M primary amine in NMP	2 h	1000	1
6	Wash	NMP DMF	30 s 30 s	2000 2000	2 1

Benzylamine is the most reliable primary amine, and a tetramer synthesized using only benzylamine submonomers had few incomplete products: benzylamine-only containing tetrameric peptoid. Peptoids consisting of benzylamine submonomers only are not water soluble to any appreciable degree, but if methoxyethylamine submonomers are used, water solubility is afforded without a drop in yield. More limited substitution is possible with aniline and forms of substituted aniline like parabromoaniline, parachloroaniline, and orthonitroaniline.

Following the synthesis of an exclusively methoxyethylamine substituted tetramer peptoid, a portion of the resin was removed from the synthesis vessel and the peptoid was cleaved and submitted for analysis. The resulting peptoid was largely free from impurity and was found at the expected molecular weight. The remaining portion of the resin was reserved in the synthesis vessel and subsequently acetylated to attach a pendant maleimido group.

To prepare a maleimido-modified peptoid, approximately 200 mg of rink amide MBHA resin (Novabiochem, La Jolla, CA) bearing the desired peptoid, 1600 μL of a 1.2M 3-maleimidopropionic acid (97%, Aldrich Chemical Co., Milwaukee, WI), 1.2M N-hydroxybenzotriazole (HOBt, Novabiochem, La Jolla, CA) solution in N,N-dimethylformamide (DMF) is added along with an additional 400 μL of 50% v/v 1,3-diisopropylcarbodiimide (DIC, Aldrich Chemical Co., Milwaukee, WI) in DMF. This slurry is agitated with a stream of Argon for thirty minutes, drained, and a fresh charge of the same mixture is added and agitated for 30 minutes. The resulting peptoid is worked up in the same way as amine-terminated peptoids. The product is not free from contaminants, and purification using HPLC with a linear gradient acetonitrile in water with .1% trifluoroacetic acid over a C4 or C18 column can be used to isolate the desired product.

EXAMPLE 3

Photocleavable Spacer-Modified Oligonucleotides

In order to test the feasibility of PC-Spacer incorporation (Glen Research, Sterling, VA), several oligonucleotides were produced by the Caltech biopolymer synthesis facility (Beckman Institute, Caltech, Pasadena, CA). Both DNA oligonucleotides contain a stretch of 25 nucleic acids complementary to a gene in *Arabadopsis thaliana* called APETALA2, and are terminated on their 5' ends by a C6 Thiol modifier (Glen Research, Sterling, VA). One of the oligonucleotides contains the PC spacer between the coding sequence and the C6 thiol, the other does not. Mass spectrometry agrees with the calculated molecular weights. Both of these are produced in identical yield as determined by measuring the OD_{260} of the product oligonucleotides in DNAse-free water (Gibco, Gaithersburg, MD). Molecular

weights and molar extinction coefficients are listed in Table 4; X is the 5' thiol cap, and Y is the photocleavable spacer. Deconvoluted ESI spectrum of 5'XCTGTTTCCGGCGGCTGAGAACCACC3', FW=8057.5, where X is the 5' thiol modifier, is different from the deconvoluted ESI spectrum of 5'XYCTGTTTCCGGCGGCTGAGAACCACC3', FW=8400.8, where X is the 5' thiol modifier and Y is the PC-spacer.

TABLE 4

Species	Molecular Weight	Molar Extinction Coefficient (/mM/cm)
5'XCTGTTTCCGGCGGCTGAGAACCACC3'	8057.5	254.3
5'XYCTGTTTCCGGCGGCTGAGAACCACC3'	8400.8	250.3

Hydrogenation of 2-(1-cyclohexenyl)ethylamine In order to produce two chemically identical peptoid libraries that are isotopically shifted, the primary amine 2-(1-cyclohexenyl)ethylamine is to be both hydrogenated and deuterated to produce a tool for shifting the molecular weight of two otherwise identical peptoids by two Daltons; Saturation of 2-(1-cyclohexenyl)ethylamine with either hydrogen or deuterium provides isotopically labeled primary amines for peptoid synthesis). Furthermore, multiple substitutions of the saturated product can be used to generate shifts of two, four, six, etc., for "multicolor" applications. Although the olefin primary amine is available commercially (Aldrich Chemical Co., Milwaukee, WI), the saturated products are not.

Approximately 300 mg of 2-(1-cyclohexenyl)ethylamine is dissolved in 10 mL of dichloromethane (EM Industries, Gibbstown, NJ) along with an additional 300 mg of Palladium on activated carbon (10% by weight, Aldrich Chemical Co., Milwaukee, WI). This mixture is stirred and occasionally sparged by hydrogen gas under atmospheric pressure. After three hours, an aliquot of the mixture is filtered over Celite (EM Industries, Gibbstown, NJ). The solvent is removed under vacuum and replaced with deuterated benzene (Cambridge Isotopes, Andover, MA) and taken for solution-phase nuclear magnetic resonance (NMR). Results suggest that this mild

treatment is able to eliminate the signature of vinyl hydrogens, and that pressurized hydrogenation or more elaborate catalysts will not be necessary.

EXAMPLE 5

On-line Disulfide-based Synthesis

The first method undertaken to join a peptoid to a DNA oligonucleotide was an on-line, DNA first method. First, DNA oligonucleotides were synthesized. These consisted only of thymine residues, because they do not require protecting groups and are likely to be more robust to a straightforward peptoid synthesis. Poly-dT_{10} was produced by the Caltech synthesis facility, and delivered directly without work-up. The 5' end of the oligomer was successively modified with a disulfide containing phosphoramidite and a 5' amine terminator. The polymer beads were removed from the robotic synthesis cartridge and loaded into the peptoid synthesis vessel. A standard round of peptoid synthesis was run to attach a single benzylamine residue to a bromoacetylated DNA oligonucleotide. This led to a very low yield of the desired product and a large quantity of difficult to identify side products as indicated by MALDI-TOF mass spectrometry (results not shown).

EXAMPLE 6

Photocleavable Peptoid_Before attempting to make use of the PC-spacer phosphoramidite, the same orthonitrobenzene moiety was incorporated into a peptoid to determine if it is capable of cleaving the neighboring amide bond when photoactivated. Incorporation of the orthonitroaniline submonomer was poor, and the resulting product did not seem to degrade extensively when exposed to longwave UV light.

Conjugate Synthesis As soon as the methyoxyethylamine substituted peptoid tetramers with the maleimido modification have been purified, they can be conjugated to the DNA oligonucleotides with 5' thiol modifications according to the method of Barton and Vreeland ((2000) Polymer Preprints 41:1018-1019. The oligonucleotides are delivered from the synthesis facility with a trityl group blocking the thiol. In order to provide the reducing conditions that will discourage DNA dimerization and remove

the trityl group, 12.8 nmol of oligonucleotide is dissolved in 30 μL of 1X triethylammonium acetate buffer (Calbiochem, La Jolla, CA) and 4.33 μL of 1M silver nitrate (Calbiochem, La Jolla, CA) and incubated for 30 minutes. To this, 5.78 mL of 1M dithiothreitol (DTT, or Cleland's Reagent, Calbiochem, La Jolla, CA) is added, and the liquid phase is aspirated and retained. The precipitate Ag-DTT is washed several times, and the supernatant liquids are combined and filtered using Centri-Spin 20 columns (Princeton Separations, Princeton, NJ) to remove buffer salts. This eluent is then frozen and lyophilized immediately to prevent dimerization. The maleimido-modified peptoid is prepared at a concentration of 12.8 mM in 0.1M sodium phosphate and 0.15 M NaCl buffer at pH=7.2. Ten mL of the peptoid solution is then added to the lyophilized DNA prepared previously, and incubated for 20 h at room temperature. The product of this reaction will be analyzed by both ESI and MALDI mass spectrometry to determine its efficiency. A fraction of the resulting probe will then be exposed to long-wave ultraviolet light for five minutes, and the analysis repeated to estimate the efficiency of cleavage. In order to produce larger numbers of these tags, the assistance of a research group at Northwestern University led by Annelise Barron that has developed a robotic synthesizer for peptoids may be sought.

Isotopic Tag Production After the hydrogenation of 2-(1-cyclohexenyl)ethylamine is repeated on a larger scale to produce several grams of the hydrogenated product, cyclohexylethylamine, the efficiency of its incorporation into peptoids can be tested. This is expected to be high, since primary amines of similar structure such as cyclohexylamine are reportedly substituted efficiently. At the same time, the saturation reaction will be repeated but with deuterium gas instead of hydrogen gas. There is no reason to expect that this reaction would work as well as it does with hydrogen. Once the tags have been produced in sufficient quantity, they can be incorporated into two chemically identical peptoids with different molecular weights. These peptoids will be made into stock solutions of known concentration, and serial dilutions will be performed of mixtures of the two solutions to test the dynamic range, sensitivity, accuracy, and reproducibility of the isotopic tag method using shared facility ESI, MALDI, and available LC-MS devices.

EXAMPLE 6

Hybridization Efficacy

In order to show method efficacy, control oligonucleotides or cloned plasmids of known concentration can be assayed for in the presence of distractor, control nucleic acids. The method for MAGE will be followed and a single nucleic acid target will be the target. The purpose of this experiment will be to determine the level of background noise, and estimate the accuracy of the assay when it is assembled as a whole. The separation method used to eliminate unhybridized probes will most likely be a poly-T resin. Following this, probes will be cleaved and the mixture will be immediately submitted for mass spectrometric analysis without further separation. The peptoid tags will be considerably smaller than the nucleic acids, and its mass is known, so the rest of the spectra can be ignored. The isotopically shifted version of the peptoid tag being used will be added as an internal standard to determine the abundance of the peptoid mass tag, and thus the target nucleic acid. Once this first experiment is complete, the version of MAGE that makes use of the selector tags will be implemented to try to boost the signal-to-noise ratio of the data generated.

EXAMPLE 7

Sensitivity Serial dilutions of either a control nucleic acid in a mixture or purified mRNA from cell culture will be subjected to the MAGE assay. By doing this, the lower limit of the MAGE assay, at least with the mass spectrometers we have easily available, will be determined. More importantly, the reliability of the data as a function of sample size can be determined.

Direct Probing of Cell Lysate Three identical MAGE experiments will be run, on two aliquots from the same cell culture. All will seek to measure the abundance of a particular gene transcript, but one will make use of purified mRNA, one total RNA, and the final, cell lysate. The signal quality and reproducibility will be compared to determine if MAGE can accept unprocessed cell lysate as starting material for measuring gene expression.

Three or More Simultaneous Conditions Chemically identical peptoid tags will be produced at three or more molecular weights using multiple substitutions of

the isotopically labeled cyclohexylethylamine. The abundance of a single gene will be measured in as many samples as there are peptoid tags of different mass but identical structure. This would, for instance, greatly reduce the work that goes into measuring a timecourse of gene expression using standard methods like RNase protection assays or real-time PCR. The results could be validated with these methods, and also by running the experiment on standardized solutions of known plasmids instead of unknown RNA from cell culture.

High Frequency Gene Expression Monitoring If MAGE has low material requirements and is fairly high throughput as is expected, the frequency of measuring gene expression during an experiment can be increased significantly without prohibitively expensive reagent use. The abundance of a particular transcript could be measured every minute for an hour after some stimulus, using an easily grown model organism like *E. coli*. This would demonstrate the potential for MAGE to generate high frequency, high fidelity data invaluable to gene regulation modelers.

Multiplexing All of the above experiments can be conducted on a single or a small group of transcripts, the probes for which could be produced manually if necessary. However, it will be important to consider the extent to which MAGE can be multiplexed. If a robotic method for peptoid synthesis can be harnessed, either combinatorially or rationally, 50 to 100 probe tags using only high suitable primary amine submonomers could be produced and made into stock solutions for later conjugation with DNA oligonucleotides. This is enough probes to study interesting gene clusters in systems of interest, and experiments at this level of multiplexing would demonstrate that MAGE is capable of generating data in amounts useful to biological modelers.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

What is claimed is

1. A method of detecting a specific nucleic acid in a sample comprising:

(a) contacting the nucleic acid with a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag, in a reaction mixture under conditions that allow the first and second oligonucleotides to specifically hybridize with the nucleic acid such that the first oligonucleotide is located immediately adjacent to the second oligonucleotide, thereby forming adjacently hybridized first and second oligonucleotides;

(b) ligating the adjacently hybridized first and second oligonucleotides to form a ligated oligonucleotide; and

(c) identifying the detector tag associated with the ligated oligonucleotide.

thereby detecting a specific nucleic acid in a sample.

2. The method of claim 1, wherein identifying the detector tag associated with the ligated oligonucleotide comprises separating, using the selector tag, the ligated oligonucleotide from the reaction mixture removing the detector tag from the ligated oligonucleotide; and identifying the detector tag associated with the ligated oligonucleotide.

3. The method of claim 2, wherein using the selector tag comprises contacting the selector tag with an agent that specifically binds to the selector tag.

4. The method of claim 2, wherein removing the detector tag is performed by subjecting the ligated oligonucleotide to a de-linking agent selected from the group consisting of an acid condition, an alkaline condition, a visible light radiation, a UV radiation, heat, a reducing condition and an oxidizing condition.

5. The method of claim 1, wherein identifying the detector tag associated with a ligated oligonucleotide comprises using mass spectrometry.

6. The method of claim 5, further comprising using chromatography.

7. The method of claim 1, wherein the selector tag is selected from a fluorescent moiety, an antibody and biotin.

8. The method of claim 1, wherein the detector tag is a peptoid.

9. A method of detecting a plurality of specific nucleic acids in a sample comprising:

(a) contacting each specific nucleic acid with an oligonucleotide pair in a reaction mixture under conditions that allow the oligonucleotide pair to specifically hybridize to the nucleic acid such that the oligonucleotide pair members are located immediately adjacent to each other thereby forming an adjacently hybridized oligonucleotides pair, wherein each oligonucleotide pair comprises a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag;

(b) ligating each adjacently hybridized oligonucleotide pair to form one or more ligated oligonucleotides; and

(c) identifying the one or more detector tags associated with the one or more ligated oligonucleotides.

thereby detecting a plurality of specific nucleic acids in a sample.

10. The method of claim 9, wherein identifying the detector tag associated with the ligated oligonucleotide comprises separating, using the selector tag, the ligated oligonucleotide from the reaction mixture, removing the detector tag from the ligated oligonucleotide; and identifying the detector tag associated with the ligated oligonucleotide.

11. The method of claim 10 wherein using the selector tag comprises contacting the selector tag with an agent that specifically binds to the selector tag.

12. The method of claim 10 wherein removing the detector tag is performed by subjecting the ligated oligonucleotide to a de-linking agent selected from the group consisting of an acid condition, an alkaline condition, a visible light radiation, a UV radiation, heat, a reducing condition and an oxidizing condition.

13. The method of claim 9, wherein identifying the detector tag associated with a ligated oligonucleotide comprises using mass spectrometry.

14. The method of claim 13 further comprising using chromatography.

15. The method of claim 9, wherein the selector tag is selected from a fluorescent moiety, an antibody and biotin.

16. The method of claim 9, wherein the detector tag is a peptoid.

17. The method of claim 9, wherein each first oligonucleotide linked to selector tag has an identical selector tag.

18. The method of claim 9, wherein each first oligonucleotide linked to selector tag has a different tag.

19. The method of claim 9, wherein each second oligonucleotide linked to detector tag has a different tag.

20. A method of detecting a nucleic acid in a sample comprising:

(a) amplifying the nucleic acid with a primer pair to form a dual-tagged amplification product in a reaction mixture, wherein the primer pair is a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag; and

(b) identifying the detector tag associated with the dual-tagged amplification product,

thereby detecting the nucleic acid in a sample.

21. The method of claim 20, wherein identifying the detector tag comprises separating, using the selector tag, the amplification product from the reaction mixture prior to identifying the detector tag associated with the amplification product.

22. The method of claim 21, wherein using the selector tag comprises contacting the selector tag with an agent that specifically binds to the selector tag

23. The method of claim 20, further comprising removing the detector tag from the amplification product prior to step (b).

24. The method of claim 23, wherein removing the detector tag is performed by subjecting the ligated oligonucleotide to a de-linking agent selected from the group consisting of an acid condition, an alkaline condition, a visible light radiation, a UV radiation, heat, a reducing condition and an oxidizing condition.

25. The method of claim 20, wherein the selector tag is selected from a fluorescent moiety, an antibody and biotin.

26. The method of claim 20, wherein the detector tag is a peptoid.

27. The method of claim 20, wherein identifying the detector tag comprises using mass spectrometry.

28. A method of detecting a nucleic acid in a sample comprising:

(a) contacting the nucleic acid with an oligonucleotide linked to a detector tag under conditions that allow the oligonucleotide to specifically hybridize to the nucleic acid to form a mixture of hybridized oligonucleotide and unhybridized oligonucleotide;

(b) separating the hybridized oligonucleotide from the unhybridized oligonucleotide; and

(a) identifying the detector tag, thereby detecting the nucleic acid.

29. The method of claim 28, wherein separating comprises contacting the mixture with an agent that binds to a polyA tail.

30. The method of claim 28, wherein separating comprises contacting the mixture with an agent that binds to a 5'-capped nucleic acid.

31. A method of generating a doubled-tagged oligonucleotide duplex comprising:

(a) contacting a single-stranded nucleic acid with a first oligonucleotide linked to a selector tag and a second oligonucleotide linked to a detector tag, under conditions that allow the first and second oligonucleotides to specifically hybridize with the nucleic acid such that the first oligonucleotide is located immediately adjacent to the second oligonucleotide thereby forming an adjacently hybridized oligonucleotide;

(b) ligating the first and second oligonucleotides

thereby forming a duplex having detector and selector tags.

32. The method of claim 31, wherein the selector tag and the detector tag are selected from a fluorescent moiety, an antibody, biotin and a peptoid, wherein the selector tag and detector tag are different.

33. A kit comprising:

(a) an oligonucleotide primer pair comprising

(i) a first selector oligonucleotide linked to a selector tag;

(ii) a second selector oligonucleotide linked to a detector tag; and

(b) an agent that binds to the selector tag.

34. A kit comprising

(a) a first selector oligonucleotide linked to a selector tag;

(b) a second selector oligonucleotide linked to a detector tag; and

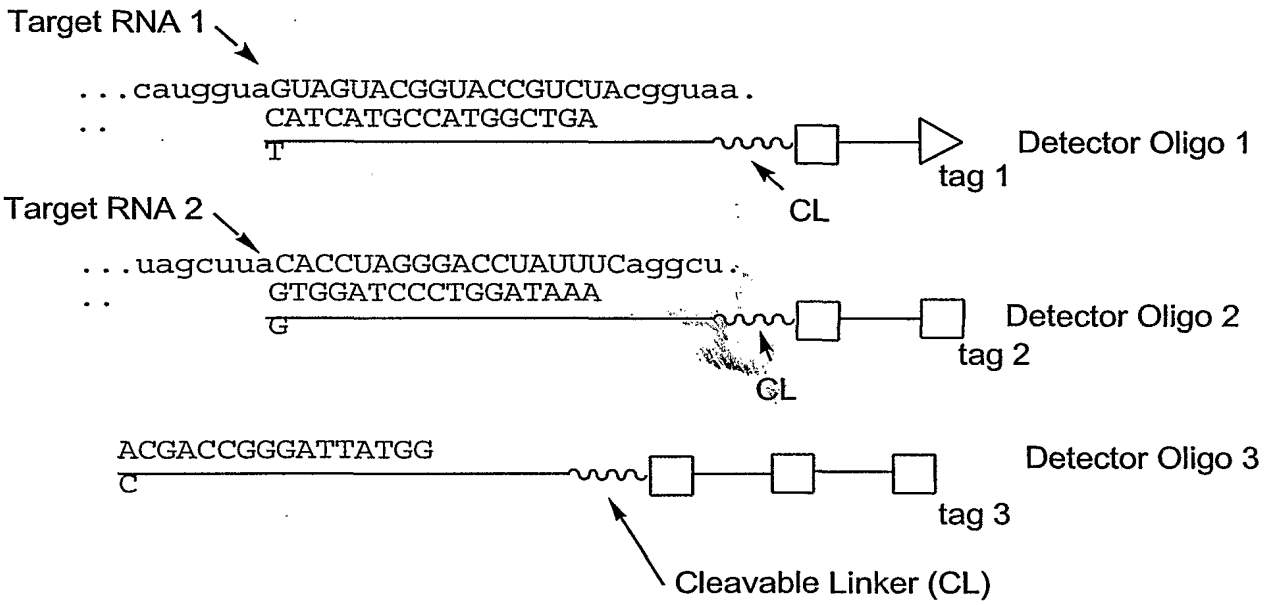
(c) a DNA ligase.

35. A library of detector oligonucleotides, comprising a plurality of oligonucleotide linked to detector tag, wherein each oligonucleotide specifically hybridizes with a nucleic acid sequence.

36. The library of claim 35, wherein the detector tag is removable.

37. The library of claim 35, wherein the detector tag is a peptoid.

38. The library of claim 35, wherein the detector tag is identifiable by mass spectroscopy.


Target RNA 1
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. .
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Detector Oligo 1
tag 1
CL
Target RNA 2
. . . uagcuuaCACCUAGGGACCUAUUUCaggcu .
. .
GTGGATCCCTGGATAAA
G
Detector Oligo 2
tag 2
CL
ACGACCGGGATTATGG
C
Detector Oligo 3
tag 3
Cleavable Linker (CL)

FIGURE 1

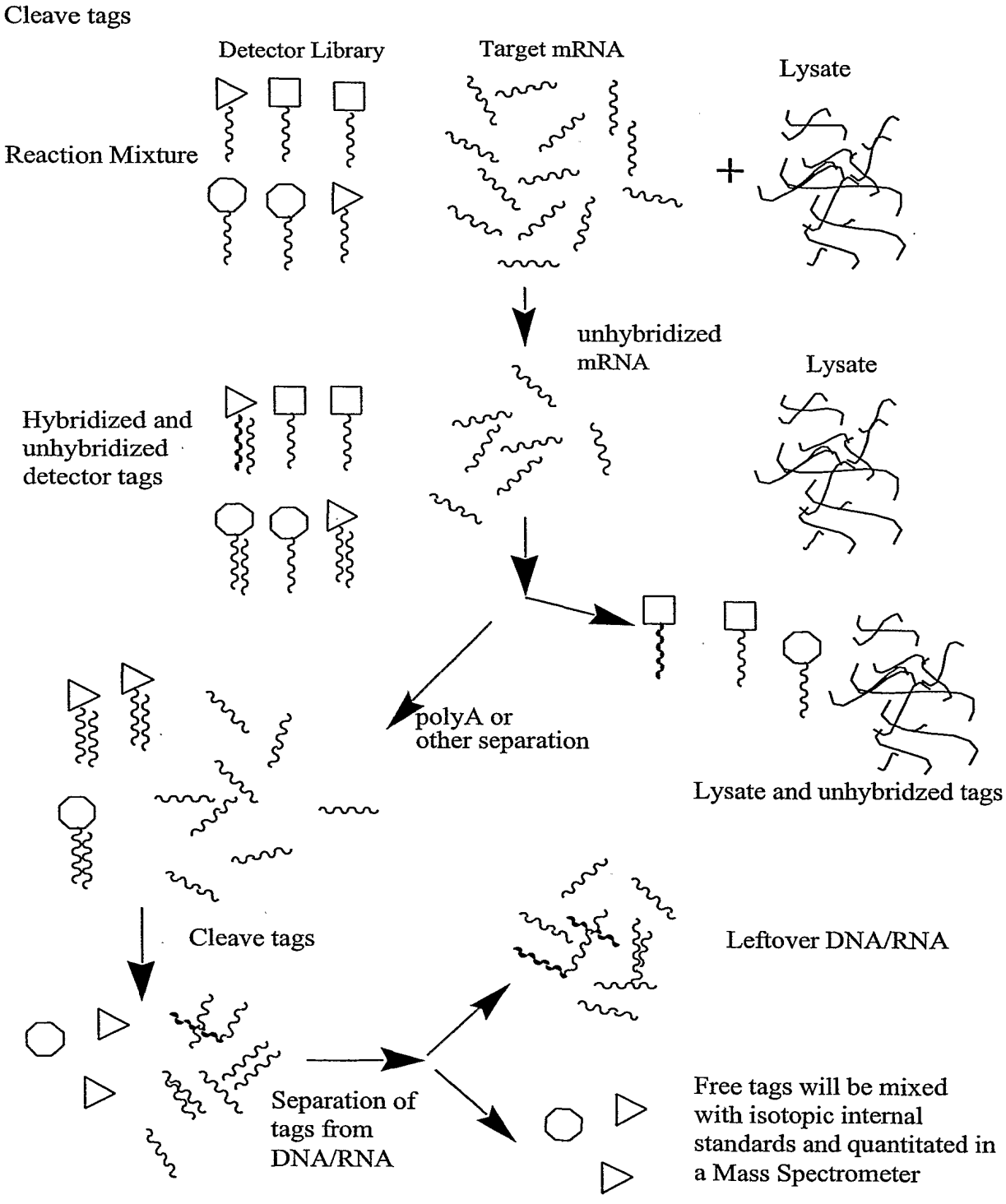

Cleave tags
Detector Library
Target mRNA
Lysate
Reaction Mixture
unhybridized
mRNA
Lysate
Hybridized and
unhybridized
detector tags
polyA or
other separation
Lysate and unhybridzed tags
Cleave tags
Leftover DNA/RNA
Separation of
tags from
DNA/RNA
Free tags will be mixed
with isotopic internal
standards and quantitated in
a Mass Spectrometer

FIGURE 2

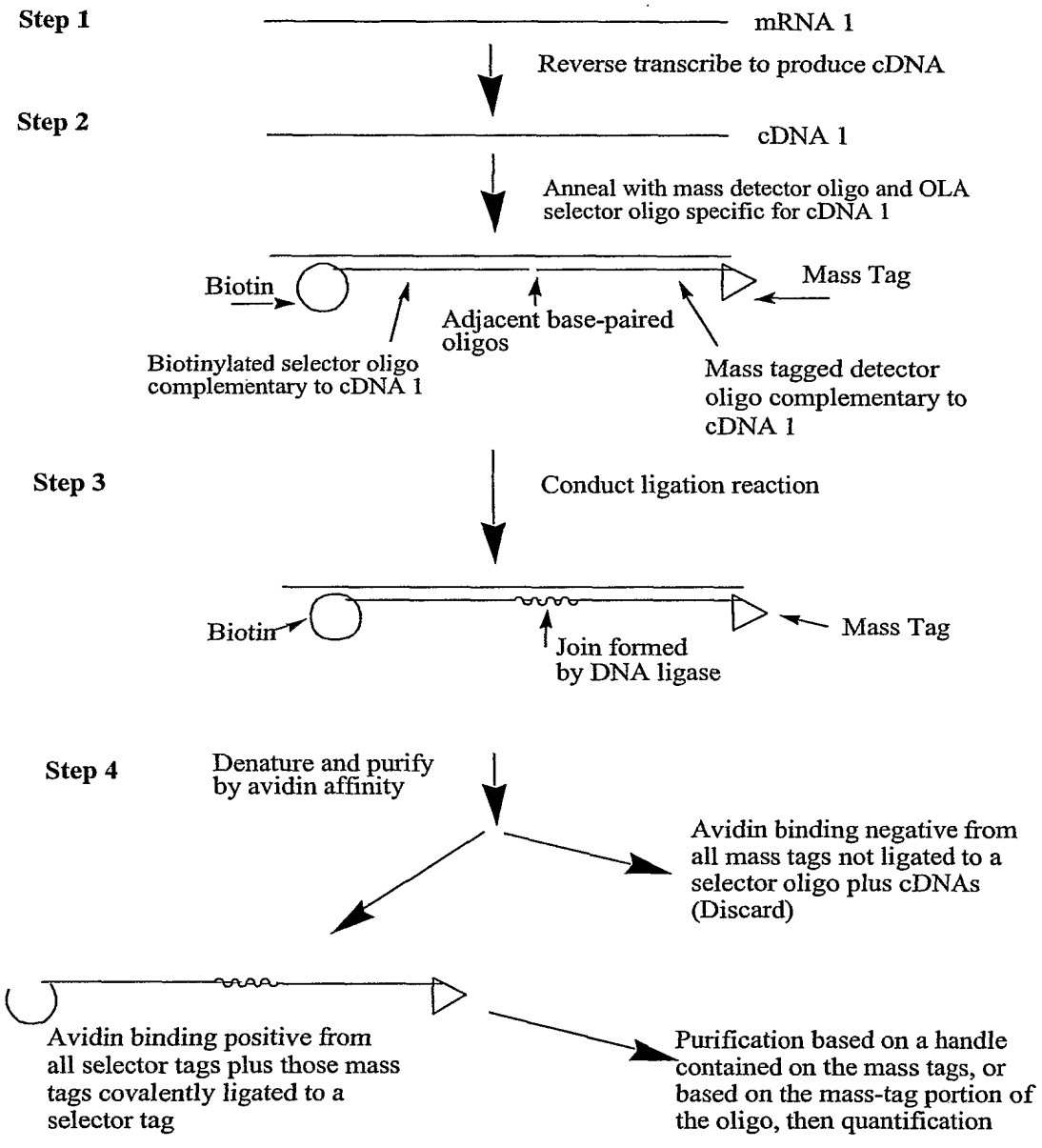

Step 1
mRNA 1
Reverse transcribe to produce cDNA
Step 2
cDNA 1
Anneal with mass detector oligo and OLA selector oligo specific for cDNA 1
Biotin
Mass Tag
Adjacent base-paired oligos
Biotinylated selector oligo complementary to cDNA 1
Mass tagged detector oligo complementary to cDNA 1
Step 3
Conduct ligation reaction
Biotin
Mass Tag
Join formed by DNA ligase
Step 4
Denature and purify by avidin affinity
Avidin binding negative from all mass tags not ligated to a selector oligo plus cDNAs (Discard)
Avidin binding positive from all selector tags plus those mass tags covalently ligated to a selector tag
Purification based on a handle contained on the mass tags, or based on the mass-tag portion of the oligo, then quantification

FIGURE 3

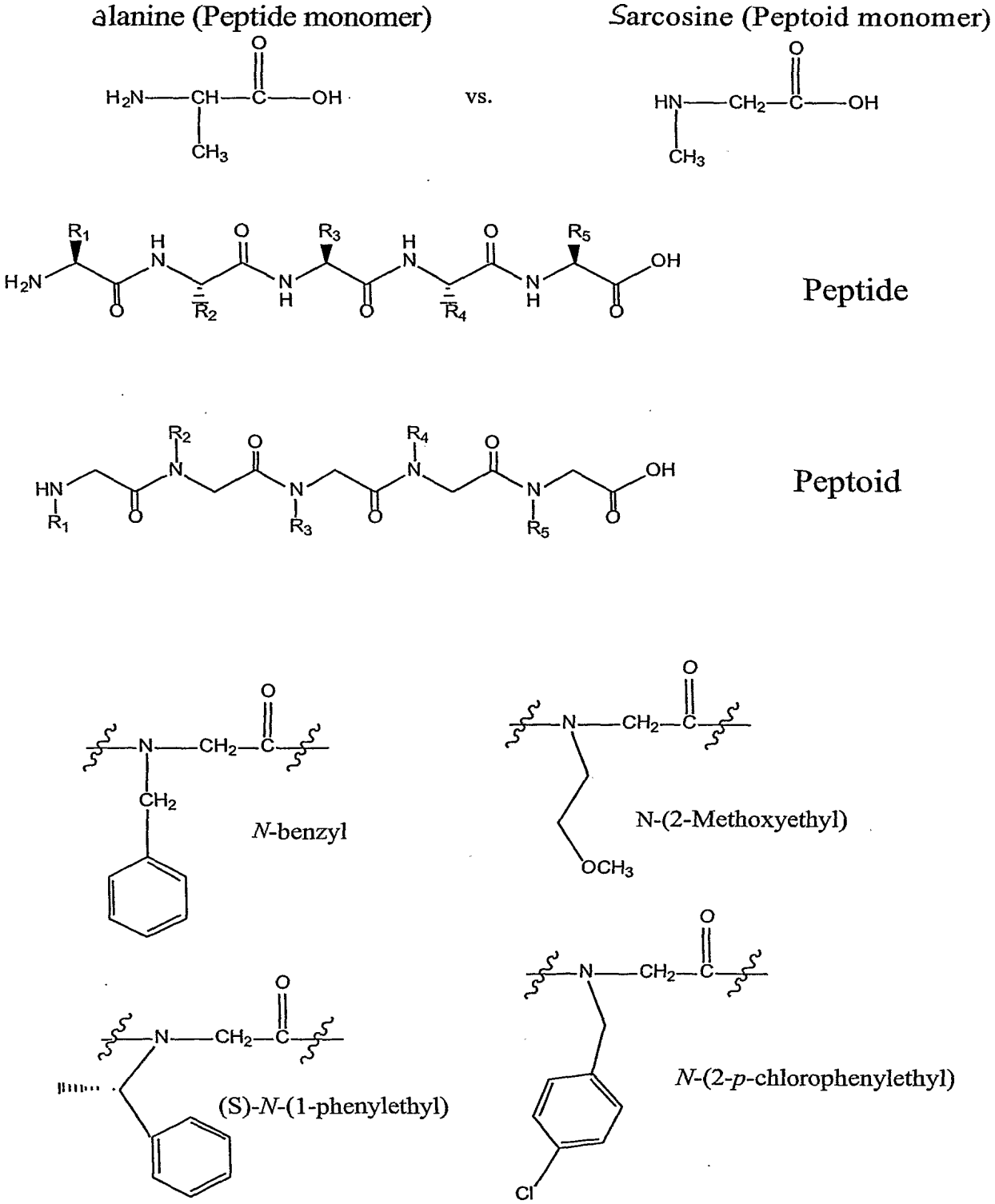

alanine (Peptide monomer)
Sarcosine (Peptoid monomer)
vs.
Peptide
Peptoid
N-benzyl
N-(2-Methoxyethyl)
(S)-N-(1-phenylethyl)
N-(2-p-chlorophenylethyl)

FIGURE 4

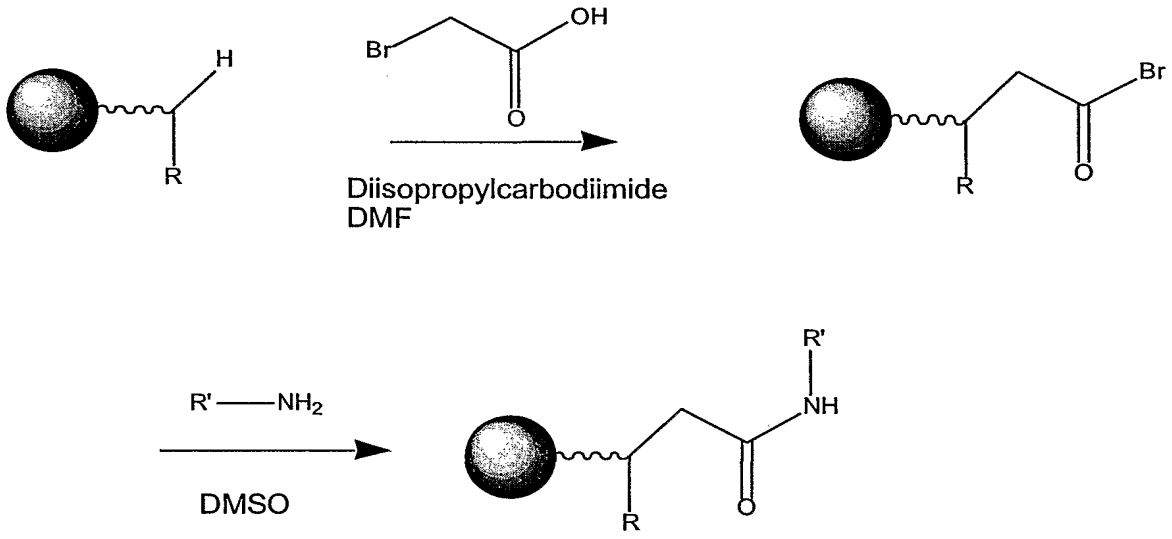
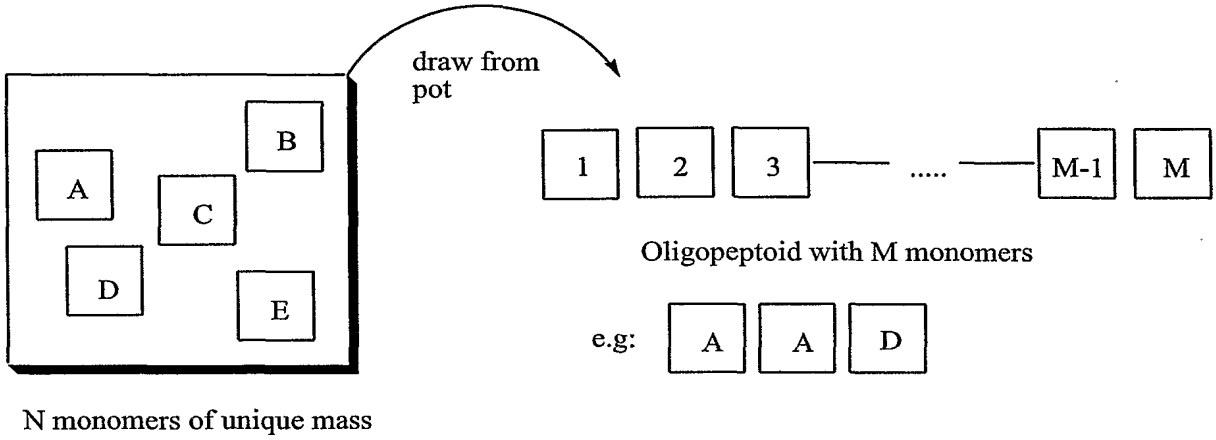

H
R
Br
OH
O
Diisopropylcarbodiimide
DMF
Br
R
O
R'—NH2
DMSO
R'
NH
R
O

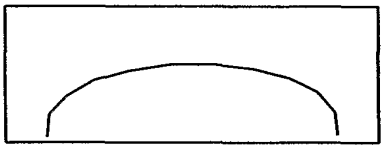
FIGURE 5



L = # of different peptoids with a unique combination of monomers

$$L = \binom{M+N-1}{N}$$

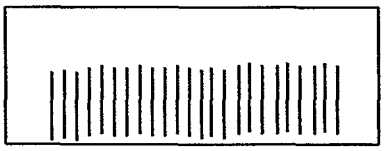
Example: 10 monomers, hexamer peptoids, yields 5005 combinations, or 8007 if pentamers and fewer are used too.



Mostly uniform distribution of molecular weights

Process library by removing coincidental combinations, when two unique combinations have the same mass

Also enforce other requirements, such as a minimum of X Daltons between species, or structural requirements such as at least two charged bases.



Desirable distribution of molecular weights

Assign oligo probes to tags and log, output library structure to peptoid synthesizer

FIGURE 6

Figure 7

N \ M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
3	3	6	10	15	21	28	36	45	55	66	78	91	105	120	136	153	171	190	210	231	253	276	300	325	351
4	4	10	20	35	56	84	120	165	220	286	364	455	560	680	816	969	1140	1330	1540	1771	2024	2300	2600	2925	3276
5	5	15	35	70	126	210	330	495	715	1001	1365	1820	2380	3060	3876	4845	5985	7315	8855	10626	12650	14950	17550	20475	23751
6	6	21	56	126	252	462	792	1287	2002	3003	4368	6188	8568	11628	15504	20349	26334	33649	42504	53130	65780	80730	98280	118755	142506
7	7	28	84	210	462	924	1716	3003	5005	8008	12376	18564	27132	38760	54264	74613	100947	134596	177100	230230	296010	376740	475020	593775	736281
8	8	36	120	330	792	1716	3432	6435	11440	19448	31824	50388	77520	116280	170544	245157	346104	480700	657800	888030	1184040	1560780	2035800	2629575	3365856
9	9	45	165	495	1287	3003	6435	12870	24310	43758	75582	125970	203490	319770	490314	735471	1081575	1562275	2220075	3108105	4292145	5852925	7888725	1.1E+07	1.4E+07
10	10	55	220	715	2002	5005	11440	24310	46620	92378	167960	293930	497420	817190	1307504	2042975	3124550	4686825	6906900	1E+07	1.4E+07	2E+07	2.8E+07	3.9E+07	5.2E+07
11	11	66	286	1001	3003	8008	19448	43758	92378	184756	352716	646646	1144066	1961256	3268760	5311735	8436285	1.3E+07	2E+07	3E+07	4.4E+07	6.5E+07	9.3E+07	1.3E+08	1.8E+08
12	12	78	364	1365	4368	12376	31824	75582	167960	352716	705432	1352078	2496144	4457400	7726160	1.3E+07	2.1E+07	3.5E+07	5.5E+07	8.5E+07	1.3E+08	1.9E+08	2.9E+08	4.2E+08	6E+08
13	13	91	455	1820	6188	18564	50388	125970	293930	646646	1352078	2704156	5200300	9657700	17383860	3E+07	5.2E+07	8.6E+07	1.4E+08	2.3E+08	3.5E+08	5.5E+08	8.3E+08	1.3E+09	1.9E+09
14	14	105	560	2380	8568	27132	77520	203490	497420	1144066	2496144	5200300	10400600	20058300	37442160	6.8E+07	1.2E+08	2.1E+08	3.5E+08	5.7E+08	9.3E+08	1.5E+09	2.3E+09	3.6E+09	5.4E+09
15	15	120	680	3060	11628	38760	116280	319770	817190	1961256	4457400	9657700	20058300	40116600	77558760	1.5E+08	2.7E+08	4.7E+08	8.2E+08	1.4E+09	2.3E+09	3.8E+09	6.1E+09	9.7E+09	1.5E+10
16	16	136	816	3876	15504	54264	170544	490314	1307504	3268760	7726160	17363860	37442160	77558760	1.55E+08	3E+08	5.7E+08	1E+09	1.9E+09	3.2E+09	5.0E+09	9.4E+09	1.5E+10	2.5E+10	4E+10
17	17	153	969	4845	20349	74613	245157	735471	2042975	5311735	13037895	30421755	67863915	1.45E+08	3.01E+08	6E+08	1.2E+09	2.2E+09	4.1E+09	7.3E+09	1.3E+10	2.2E+10	3.8E+10	6.3E+10	1E+11
18	18	171	1140	5985	26334	100947	346104	1081575	3124550	8436285	21474180	51895935	1.2E+08	2.65E+08	5.66E+08	1.2E+09	2.3E+09	4.5E+09	8.6E+09	1.6E+10	2.9E+10	5.1E+10	8.9E+10	1.5E+11	2.5E+11
19	19	190	1330	7315	33649	134596	480700	1562275	4686825	13123110	34597290	86493225	2.06E+08	4.71E+08	1.04E+09	2.2E+09	4.5E+09	9.1E+09	1.8E+10	3.4E+10	6.2E+10	1.1E+11	2E+11	3.5E+11	6.1E+11
20	20	210	1540	8855	42504	177100	657800	2220075	6906900	20030010	54627300	1.41E+08	3.47E+08	8.19E+08	1.86E+09	4.1E+09	8.6E+09	1.8E+10	3.5E+10	6.9E+10	1.3E+11	2.4E+11	4.5E+11	8E+11	1.4E+12
21	21	231	1771	10626	53130	230230	888030	3108105	10015005	30045015	84672315	2.26E+08	5.73E+08	1.39E+09	3.25E+09	7.3E+09	1.6E+10	3.4E+10	6.9E+10	1.4E+11	2.7E+11	5.1E+11	9.6E+11	1.8E+12	3.2E+12
22	22	253	2024	12650	65780	296010	1184040	4292145	14307150	44352165	1.29E+08	3.55E+08	9.28E+08	2.32E+09	5.57E+09	1.3E+10	2.9E+10	6.2E+10	1.3E+11	2.7E+11	5.4E+11	1.1E+12	2E+12	3.8E+12	6.9E+12
23	23	276	2300	14950	80730	376740	1560780	5852925	20160075	64512240	1.94E+08	5.48E+08	1.48E+09	3.8E+09	9.36E+09	2.2E+10	5.1E+10	1.1E+11	2.4E+11	5.1E+11	1.1E+12	2.1E+12	4.1E+12	7.9E+12	1.5E+13
24	24	300	2600	17550	98280	475020	2035800	7888725	28048800	92561040	2.86E+08	8.34E+08	2.31E+09	6.11E+09	1.55E+10	3.8E+10	8.9E+10	2E+11	4.5E+11	9.6E+11	2E+12	4.1E+12	8.2E+12	1.6E+13	3.1E+13
25	25	325	2925	20475	118755	593775	2629575	10518300	38567100	1.31E+08	4.17E+08	1.25E+09	3.56E+09	9.67E+09	2.51E+10	6.3E+10	1.5E+11	3.5E+11	8E+11	1.8E+12	3.8E+12	7.9E+12	1.6E+13	3.2E+13	6.3E+13
26	26	351	3276	23751	142506	736281	3365856	13884156	52451256	1.84E+08	6.01E+08	1.85E+09	5.41E+09	1.51E+10	4.02E+10	1E+11	2.65E+11	6.1E+11	1.4E+12	3.2E+12	6.9E+12	1.5E+13	3.1E+13	6.3E+13	1.3E+14
27	27	378	3654	27405	169911	906192	4272048	18156204	70607460	2.54E+08	8.55E+08	2.71E+09	8.12E+09	2.32E+10	6.34E+10	1.7E+11	4.2E+11	1E+12	2.4E+12	5.6E+12	1.3E+13	2.7E+13	5.8E+13	1.2E+14	2.5E+14
28	28	406	4060	31465	201376	1107568	5379616	23536820	94143280	3.46E+08	1.2E+09	3.91E+09	1.2E+10	3.52E+10	9.87E+10	2.7E+11	6.9E+11	1.7E+12	4.2E+12	9.8E+12	2.2E+13	5E+13	1.1E+14	2.3E+14	4.8E+14
29	29	435	4495	35960	237336	1344904	6724520	30260340	1.24E+08	4.73E+08	1.68E+09	5.59E+09	1.76E+10	5.29E+10	1.52E+11	4.2E+11	1.1E+12	2.8E+12	7E+12	1.7E+13	3.9E+13	8.9E+13	2E+14	4.3E+14	9E+14
30	30	465	4960	40920	278256	1623160	8347680	38608020	1.63E+08	6.36E+08	2.31E+09	7.9E+09	2.55E+10	7.84E+10	2.3E+11	6.5E+11	1.7E+12	4.6E+12	1.2E+13	2.8E+13	6.7E+13	1.6E+14	3.8E+14	7.8E+14	1.7E+15
31	31	496	5456	46376	324632	1947792	10295472	48903492	2.12E+08	8.48E+08	3.16E+09	1.11E+10	3.66E+10	1.15E+11	3.45E+11	9.9E+11	2.7E+12	7.3E+12	1.9E+13	4.7E+13	1.1E+14	2.7E+14	6.2E+14	1.4E+15	3.1E+15
32	32	528	5984	52360	376992	2324784	12620256	61523748	2.73E+08	1.12E+09	4.28E+09	1.53E+10	5.19E+10	1.67E+11	5.12E+11	1.5E+12	4.2E+12	1.2E+13	3E+13	7.8E+13	1.9E+14	4.6E+14	1.1E+15	2.5E+15	5.6E+15
33	33	561	6545	58905	435897	2760681	15386937	76904685	3.5E+08	1.47E+09	5.76E+09	2.11E+10	7.3E+10	2.4E+11	7.52E+11	2.3E+12	6.5E+12	1.8E+13	4.8E+13	1.3E+14	3.2E+14	7.8E+14	1.9E+15	4.4E+15	9.9E+15
34	34	595	7140	66045	501942	3262623	18643560	95548245	4.46E+08	1.92E+09	7.67E+09	2.88E+10	1.02E+11	3.42E+11	1.09E+12	3.3E+12	9.9E+12	2.8E+13	7.6E+13	2E+14	5.2E+14	1.3E+15	3.2E+15	7.5E+15	1.7E+16
35	35	630	7770	73815	575757	3838380	22481940	1.18E+08	5.64E+08	2.48E+09	1.02E+10	3.89E+10	1.41E+11	4.82E+11	1.58E+12	4.9E+12	1.5E+13	4.3E+13	1.2E+14	3.2E+14	8.4E+14	2.1E+15	5.3E+15	1.3E+16	3E+16
36	36	666	8436	82251	658008	4496388	26976328	1.45E+08	7.09E+08	3.19E+09	1.35E+10	5.23E+10	1.93E+11	6.75E+11	2.25E+12	7.2E+12	2.2E+13	6.5E+13	1.8E+14	5.1E+14	1.3E+15	3.5E+15	8.8E+15	2.2E+16	5.2E+16
37	37	703	9139	91390	749398	5245786	32224114	1.77E+08	8.86E+08	4.08E+09	1.74E+10	6.97E+10	2.63E+11	9.38E+11	3.19E+12	1E+13	3.2E+13	9.7E+13	2.8E+14	7.9E+14	2.1E+15	5.6E+15	1.4E+16	3.6E+16	8.8E+16
38	38	741	9880	101270	850668	6096454	38320568	2.16E+08	1.1E+09	5.18E+09	2.26E+10	9.23E+10	3.55E+11	1.29E+12	4.48E+12	1.5E+13	4.7E+13	1.4E+14	4.2E+14	1.2E+15	3.3E+15	9E+15	2.3E+16	5.9E+16	1.5E+17
39	39	780	10660	111930	962598	7059052	45379620	2.61E+08	1.36E+09	6.54E+09	2.91E+10	1.21E+11	4.76E+11	1.77E+12	6.25E+12	2.1E+13	6.8E+13	2.1E+14	6.4E+14	1.8E+15	5.2E+15	1.4E+16	3.8E+16	9.7E+16	2.4E+17
40	40	820	11480	123410	1086008	8145060	53524680	3.14E+08	1.68E+09	8.22E+09	3.74E+10	1.59E+11	6.35E+11	2.4E+12	8.65E+12	3E+13	9.8E+13	3.1E+14	9.5E+14	2.8E+15	8E+15	2.2E+16	6E+16	1.6E+17	4E+17

M=length of polypeptoid
N=# of monomers of unique mass

Figure 8A

N ↓ / M →	1	2	3	4	5	6	7	8	9	10	11	12
1	1	2	3	4	5	1	6	7	8	9	1	10
2	2	5	9	14	20	27	35	44	54	65	77	90
3	3	9	19	34	55	83	119	164	219	285	363	454
4	4	14	34	69	125	209	329	494	714	1000	1364	1819
5	5	20	55	125	251	461	791	1286	2001	3002	4367	6187
6	6	27	83	209	461	923	1715	3002	5004	8007	12375	18563
7	7	35	119	329	791	1715	3431	6434	11439	19447	31823	50387
8	8	44	164	494	1286	3002	6434	12869	24309	43757	75581	125969
9	9	54	219	714	2001	5004	11439	24309	48619	92377	167959	293929
10	10	65	285	1000	3002	8007	19447	43757	92377	184755	352715	646645
11	11	77	363	1364	4367	12375	31823	75581	167959	352715	705431	1352077
12	12	90	454	1819	6187	18563	50387	125969	293929	646645	1352077	2704155
13	13	104	559	2379	8567	27131	77519	203489	497419	1144065	2496143	5200299
14	14	119	679	3059	11627	38759	116279	319769	817189	1961255	4457399	9657699
15	15	135	815	3875	15503	54263	170543	490313	1307503	3268759	7726159	17383859
16	16	152	968	4844	20348	74612	245156	735470	2042974	5311734	13037894	30421754
17	17	170	1139	5984	26333	100946	346103	1081574	3124549	8436284	21474179	51895934
18	18	189	1329	7314	33648	134595	480699	1562274	4686824	13123109	34597289	86493224
19	19	209	1539	8854	42503	177099	657799	2220074	6906899	20030009	54627299	141120524
20	20	230	1770	10625	53129	230229	888029	3108104	10015004	30045014	84672314	225792839
21	21	252	2023	12649	65779	296009	1184039	4292144	14307149	44352164	129024479	354817319
22	22	275	2299	14949	80729	376739	1560779	5852924	20160074	64512239	193536719	548354039
23	23	299	2599	17549	98279	475019	2035799	7888724	28048799	92561039	286097759	834451799
24	24	324	2924	20474	118754	593774	2629574	10518299	38567099	131128139	417225899	1251677699
25	25	350	3275	23750	142505	736280	3365855	13884155	52451255	183579395	600805295	1852482995
26	26	377	3653	27404	169910	906191	4272047	18156203	70607459	254186855	854992151	2707475147
27	27	405	4059	31464	201375	1107567	5379615	23535819	94143279	348330135	1203322287	3910797435
28	28	434	4494	35959	237335	1344903	6724519	30260339	124403619	472733755	1676056043	5586853479
29	29	464	4959	40919	278255	1623159	8347679	38608019	163011639	635745395	2311801439	7898654919
30	30	495	5455	46375	324631	1947791	10295471	48903491	211915131	847660527	3159461967	11058116887
31	31	527	5983	52359	376991	2324783	12620255	61523747	273438879	1121099407	4280561375	15338678263
32	32	560	6544	58904	435896	2760680	15380936	76904684	350343564	1471442972	5752004348	21090682612
33	33	594	7139	66044	501941	3262622	18643559	95548244	445891809	1917334782	7669339131	28760021744
34	34	629	7769	73814	575756	3838379	22481939	118030184	563921994	2481256777	10150595909	38910617654
35	35	665	8435	82250	658007	4496387	26978327	145008512	708930507	3190187285	13340783195	52251400850
36	36	702	9138	91389	749397	5245785	32224113	177232626	886163134	4076350420	17417133616	69668534467
37	37	740	9879	101269	850667	6096453	38320567	215553194	1101716329	5178066750	22595200367	92263734835
38	38	779	10659	111929	962597	7059051	45379619	260932814	1362649144	6540715895	29135916263	1.21E+11
39	39	819	11479	123409	1086007	8145059	53524679	314457494	1677106639	8217822535	37353738799	1.59E+11
40	40	860	12340	135750	1221758	9366818	62891498	377348993	2054455633	10272278169	47626016969	2.06E+11

Figure 8B

N	M	13	14	15	16	17	18	19	20	21	22	23	24	25
	1	11	12	13	1	14	15	16	17	1	18	19	20	21
1	1	104	119	135	152	170	189	209	230	252	275	299	324	350
2	2	559	679	815	968	1139	1329	1539	1770	2023	2299	2599	2924	3275
3	3	2379	3059	3875	4844	5984	7314	8854	10625	12649	14949	17549	20474	23750
4	4	8567	11627	15503	20348	26333	33648	42503	53129	65779	80729	98279	118754	142505
5	5	27131	38759	54263	74612	100946	134595	177099	230229	296009	376739	475019	593774	736280
6	6	77519	116279	170543	245156	346103	480699	657799	888029	1184039	1560779	2035799	2629574	3365855
7	7	203489	319769	490313	735470	1081574	1562274	2220074	3108104	4292144	5852924	7888724	10518299	13884155
8	8	497419	817189	1307503	2042974	3124549	4686824	6906899	10015004	14307149	20160074	28048799	38567099	52451255
9	9	1144065	1961255	3268759	5311734	8436284	13123109	20030009	30045014	44352164	64512239	92561039	131128139	183579395
10	10	2496143	4457399	7726159	13037894	21474179	34597289	54627299	84672314	129024479	193536719	286097759	417225899	600805295
11	11	5200299	9657699	17383859	30421754	51895934	86493224	141120524	225792839	354817319	548354039	834451799	1251677699	1852482995
12	12	10400599	20058299	37442159	67863914	119759849	206253074	347373599	573166439	927983759	1476337799	2310789599	3562467299	5414950295
13	13	20058299	40116599	77558759	145422674	265182524	471435599	818809199	1391975639	2319959399	3796297199	6107086799	9669554099	15084504395
14	14	37442159	77558759	155117519	300540194	565722719	1037158319	1855967519	3247943159	5567902559	9364199759	15471286559	25140840659	40225345055
15	15	67863914	145422674	300540194	601080389	1166803109	2203961429	4059928949	7307872109	12875774669	22239974429	37711260989	62852101649	1.03077E+11
16	16	119759849	265182524	565722719	1166803109	2333606219	4537567649	8597496599	15905368709	28781143379	51021117809	88732378799	1.51584E+11	2.54662E+11
17	17	206253074	471435599	1037158319	2203961429	4537567649	9075135299	17672631899	33578000609	62359143989	1.1338E+11	2.02113E+11	3.53697E+11	6.08359E+11
18	18	347373599	818809199	1855967519	4059928949	8597496599	17672631899	35345263799	68923264409	1.31282E+11	2.44663E+11	4.46775E+11	8.00472E+11	1.40883E+12
19	19	573166439	1391975639	3247943159	7307872109	15905368709	33578000609	68923264409	1.37847E+11	2.69129E+11	5.13792E+11	9.60567E+11	1.76104E+12	3.16987E+12
20	20	927983759	2319959399	5567902559	12875774669	28781143379	62359143989	1.31282E+11	2.69129E+11	5.38258E+11	1.05205E+12	2.01262E+12	3.77366E+12	6.94353E+12
21	21	1476337799	3796297199	9364199759	22239974429	51021117809	1.1338E+11	2.44663E+11	5.13792E+11	1.05205E+12	2.1041E+12	4.11672E+12	7.89037E+12	1.48339E+13
22	22	2310789599	6107086799	15471286559	37711260989	88732378799	2.02113E+11	4.46775E+11	9.60567E+11	2.01262E+12	4.11672E+12	8.23343E+12	1.61238E+13	3.09577E+13
23	23	3562467299	9669554099	25140840659	62852101649	1.51584E+11	3.53697E+11	8.00472E+11	1.76104E+12	3.77366E+12	7.89037E+12	1.61238E+13	3.22476E+13	6.32053E+13
24	24	5414950295	15084504395	40225345055	1.03077E+11	2.54662E+11	6.08359E+11	1.40883E+12	3.16987E+12	6.94353E+12	1.48339E+13	3.09577E+13	6.32053E+13	1.26411E+14
25	25	8122425443	23206929839	63432274895	1.6651E+11	4.21172E+11	1.02953E+12	2.43836E+12	5.60823E+12	1.25518E+13	2.73857E+13	5.83434E+13	1.21549E+14	2.47959E+14
26	26	12033222879	35240152719	98672427615	2.65182E+11	6.86354E+11	1.71588E+12	4.15425E+12	9.76248E+12	2.23142E+13	4.96999E+13	1.08043E+14	2.29592E+14	4.77551E+14
27	27	17620076359	52860229079	1.51533E+11	4.16715E+11	1.10307E+12	2.81895E+12	6.9732E+12	1.67357E+13	3.90499E+13	8.87498E+13	1.96793E+14	4.26385E+14	9.03936E+14
28	28	25518731279	78378960359	2.29912E+11	6.46626E+11	1.7497E+12	4.56865E+12	1.15418E+13	2.82775E+13	6.73274E+13	1.56077E+14	3.5287E+14	7.79255E+14	1.68319E+15
29	29	36576848167	1.14956E+11	3.44867E+11	9.91494E+11	2.74119E+12	7.30984E+12	1.88517E+13	4.71292E+13	1.14457E+14	2.70534E+14	6.23404E+14	1.40266E+15	3.08585E+15
30	30	51915526431	1.66871E+11	5.11739E+11	1.50323E+12	4.24442E+12	1.15543E+13	3.04059E+13	7.75352E+13	1.91992E+14	4.62526E+14	1.08593E+15	2.48859E+15	5.57444E+15
31	31	73006209044	2.39878E+11	7.51616E+11	2.25485E+12	6.49927E+12	1.80535E+13	4.84595E+13	1.25995E+14	3.17986E+14	7.80512E+14	1.86644E+15	4.35503E+15	9.92947E+15
32	32	1.01766E+11	3.41644E+11	1.09326E+12	3.34811E+12	9.84738E+12	2.79009E+13	7.63604E+13	2.02355E+14	5.20341E+14	1.30085E+15	3.1673E+15	7.52233E+15	1.74518E+16
33	33	1.40677E+11	4.82321E+11	1.57558E+12	4.92369E+12	1.47711E+13	4.2672E+13	1.19032E+14	3.21387E+14	8.41729E+14	2.14258E+15	5.30988E+15	1.28322E+16	3.0284E+16
34	34	1.92928E+11	6.75249E+11	2.25083E+12	7.17452E+12	2.19456E+13	6.46176E+13	1.8365E+14	5.05037E+14	1.34677E+15	3.48935E+15	8.79923E+15	2.16314E+16	5.19154E+16
35	35	2.62597E+11	9.37846E+11	3.18868E+12	1.03632E+13	3.23088E+13	9.69263E+13	2.80576E+14	7.85614E+14	2.13238E+15	5.62173E+15	1.4421E+16	3.60524E+16	8.79678E+16
36	36	3.54861E+11	1.29271E+12	4.48138E+12	1.48446E+13	4.71534E+13	1.4408E+14	4.24656E+14	1.21027E+15	3.34265E+15	8.96438E+15	2.33853E+16	5.94377E+16	1.47406E+17
37	37	4.7626E+11	1.76897E+12	6.25035E+12	2.10949E+13	6.82483E+13	2.12328E+14	6.36984E+14	1.84725E+15	5.1899E+15	1.41543E+16	3.75396E+16	9.69773E+16	2.44383E+17
38	38	6.35014E+11	2.40398E+12	8.65433E+12	2.97493E+13	9.79975E+13	3.10326E+14	9.47309E+14	2.79456E+15	7.98447E+15	2.21387E+16	5.96784E+16	1.56656E+17	4.01039E+17
39	39	8.41393E+11	3.24537E+12	1.18997E+13	4.1649E+13	1.39646E+14	4.49972E+14	1.39728E+15	4.19184E+15	1.21763E+16	3.43151E+16	9.39934E+16	2.50649E+17	6.51688E+17
40	40													

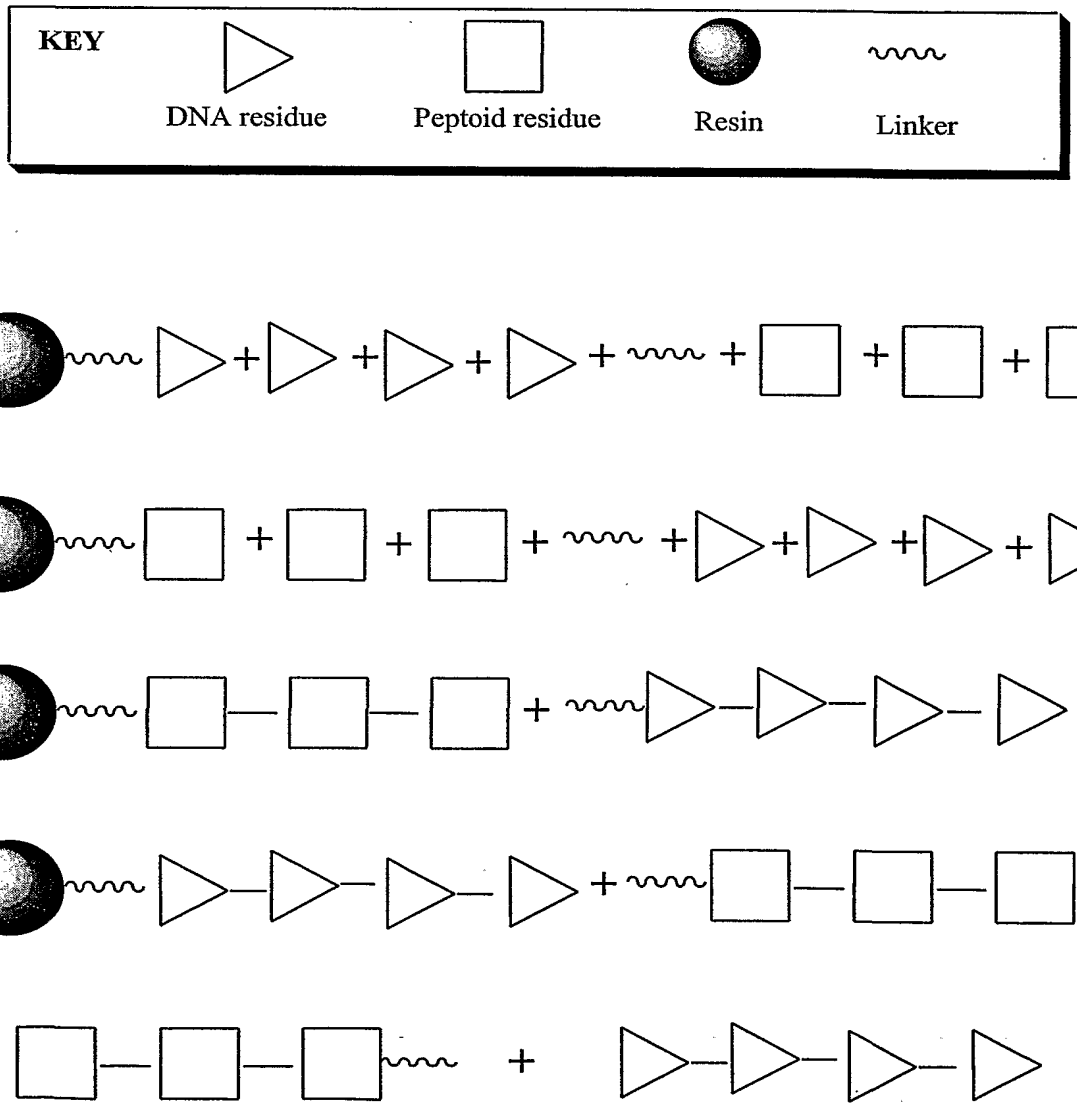


FIGURE 9

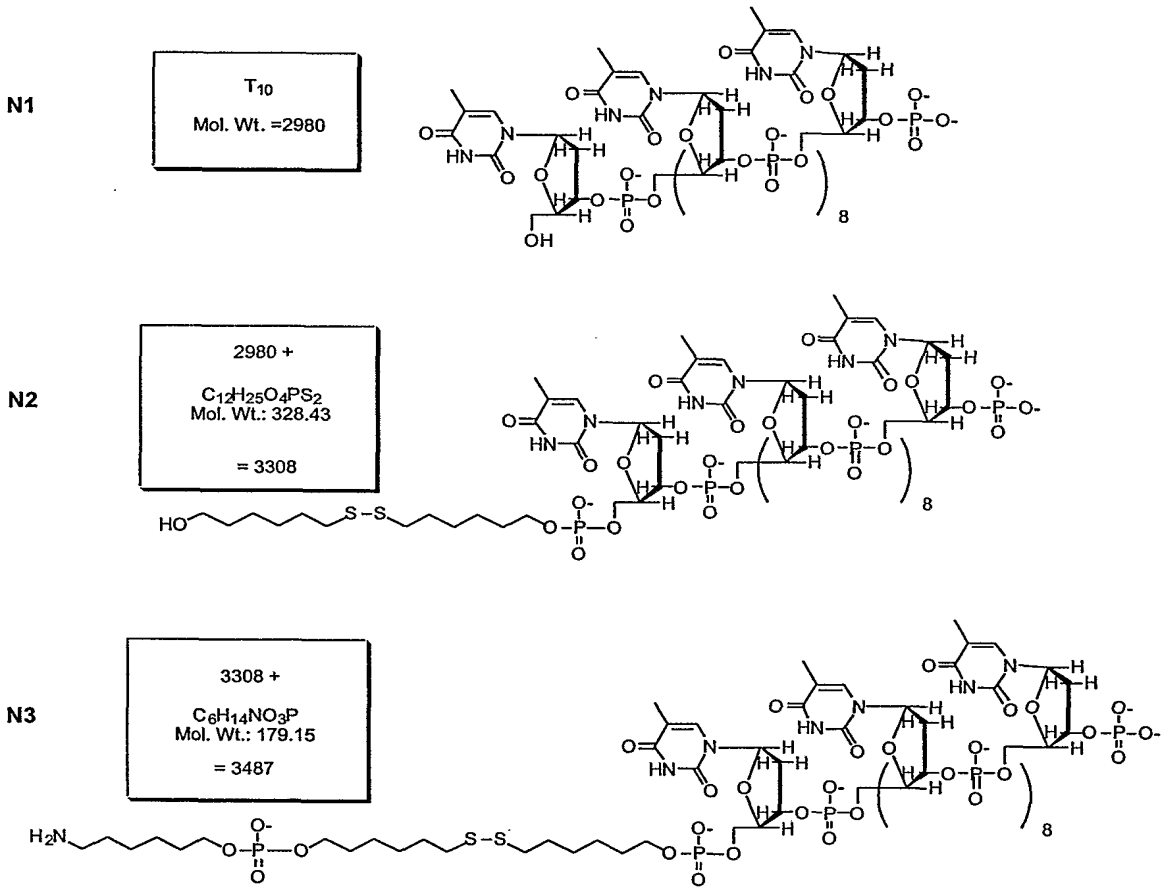

N1
T10
Mol. Wt. =2980
N2
2980 +
C12H25O4PS2
Mol. Wt.: 328.43
= 3308
N3
3308 +
C6H14NO3P
Mol. Wt.: 179.15
= 3487

FIGURE 10

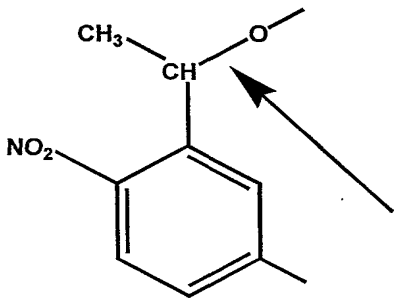


FIGURE 11A

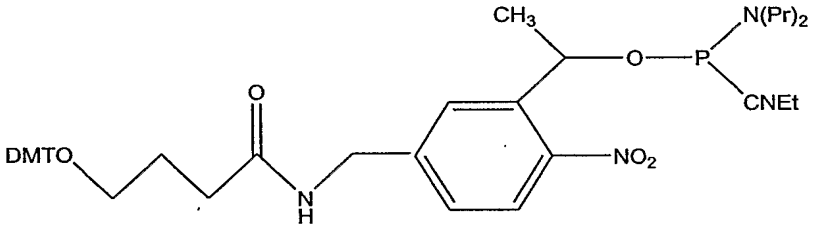


FIGURE 11B

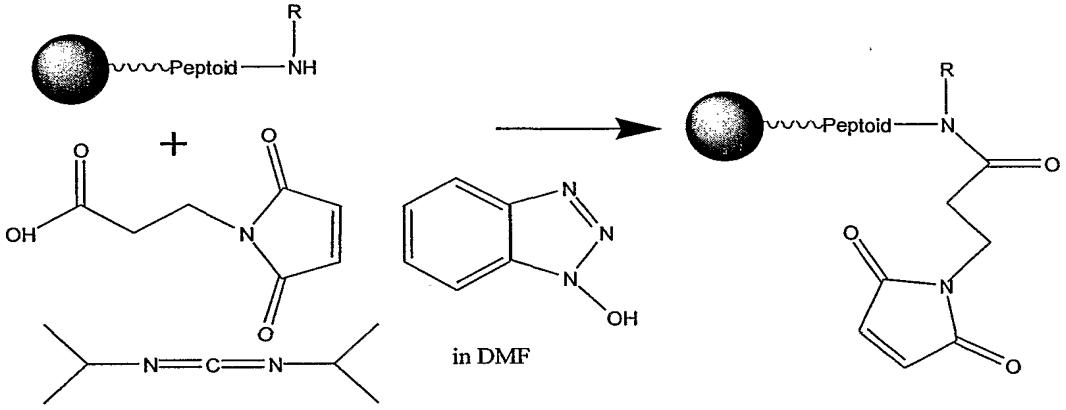


FIGURE 11C

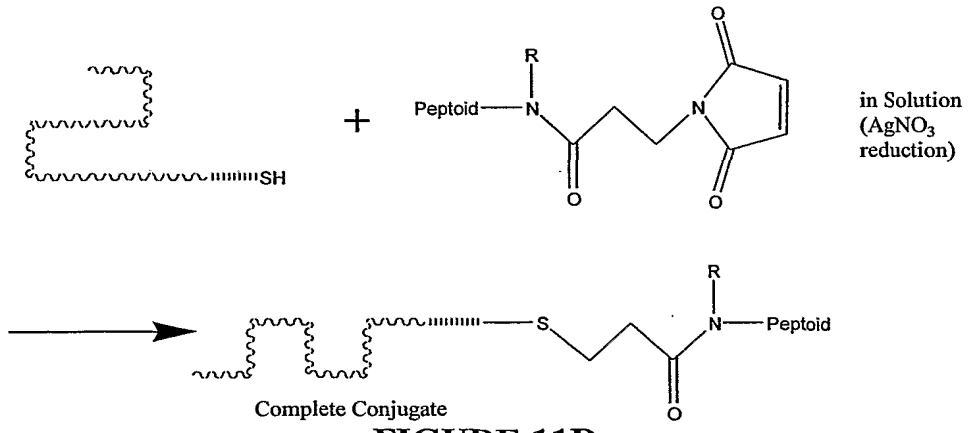


FIGURE 11D

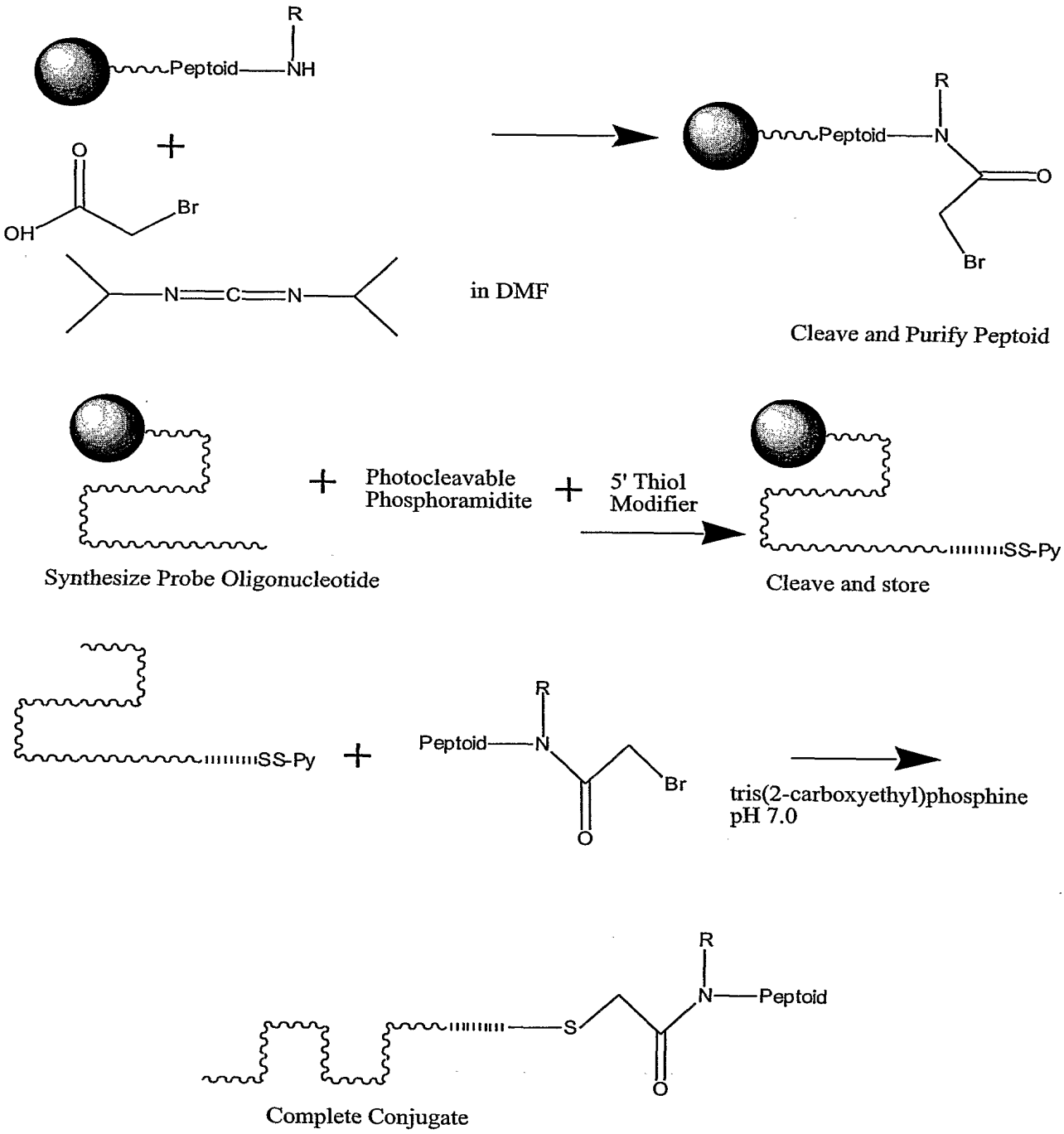
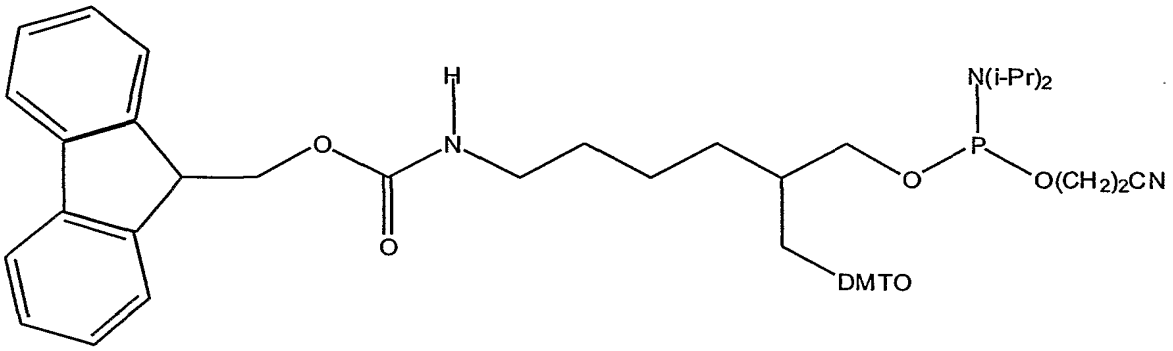

R
Peptoid
NH
O
OH
Br
N=C=N
in DMF
Peptoid
N
O
Br
Cleave and Purify Peptoid
Photocleavable Phosphoramidite
5' Thiol Modifier
SS-Py
Synthesize Probe Oligonucleotide
Cleave and store
SS-Py
Peptoid
N
Br
O
tris(2-carboxyethyl)phosphine
pH 7.0
R
N
Peptoid
S
O
Complete Conjugate

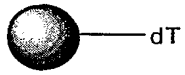
FIGURE 11E



Clontech "Uni-Link AminoModifier" Branched Phosphoramidite

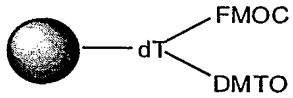
Method

1. Obtain Oligonucleotide Resin with dT base



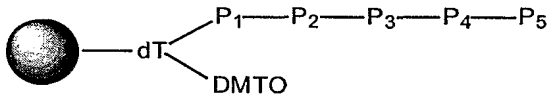
2. Add Branched Phosphoramidite

3. Transfer to Peptoid Synthesizer



4. Deprotect FMOC

5. Add Peptoid



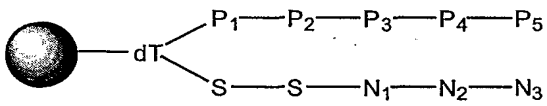
6. Protect Terminus

7. Return to ODN Synthesizer

8. Deprotect DMTO

9. Add cleavable units (Disulfide or Photocleavable)

10. Synthesize ODN



11. Deprotect and Cleave Completed Unit

FIGURE 12

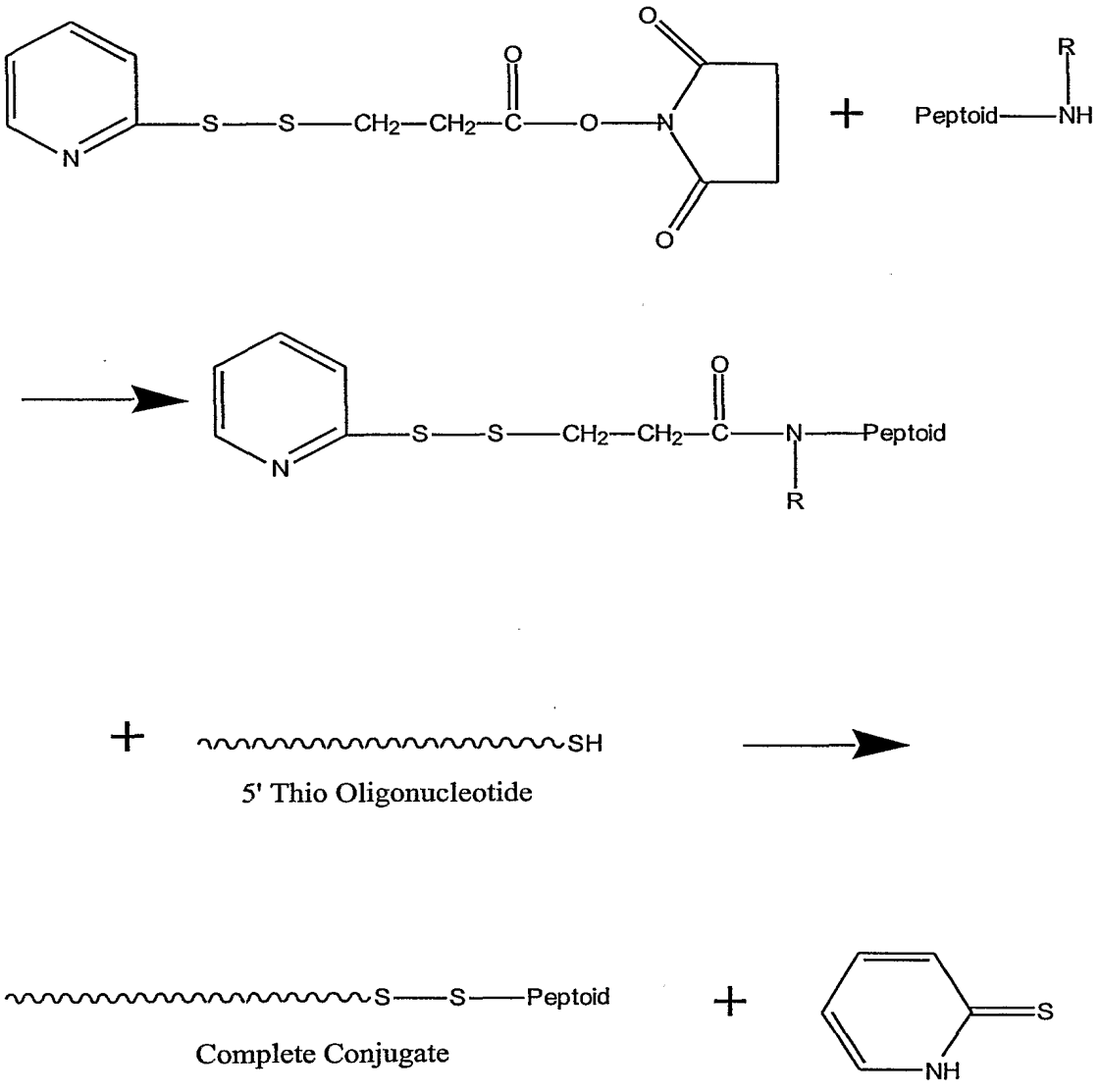

S—S—CH2—CH2—C—O—N
O
O
O
N
+
Peptoid—NH
R
S—S—CH2—CH2—C—N—Peptoid
R
+
SH
5' Thio Oligonucleotide
S—S—Peptoid
Complete Conjugate
+
S
NH

FIGURE 13

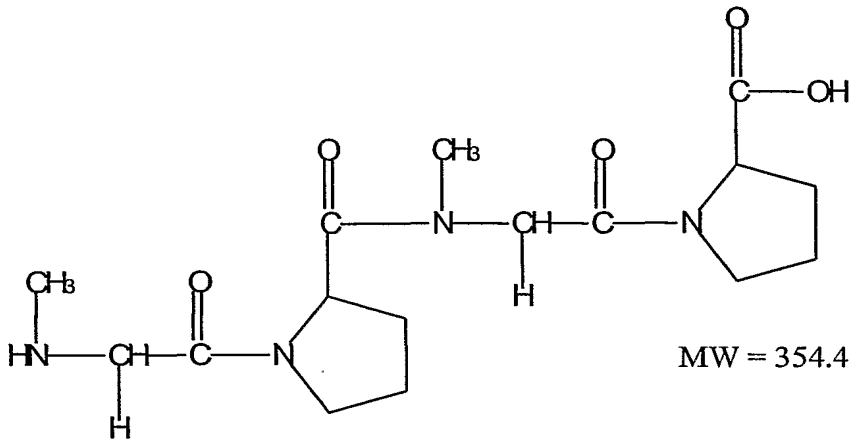


FIGURE 14

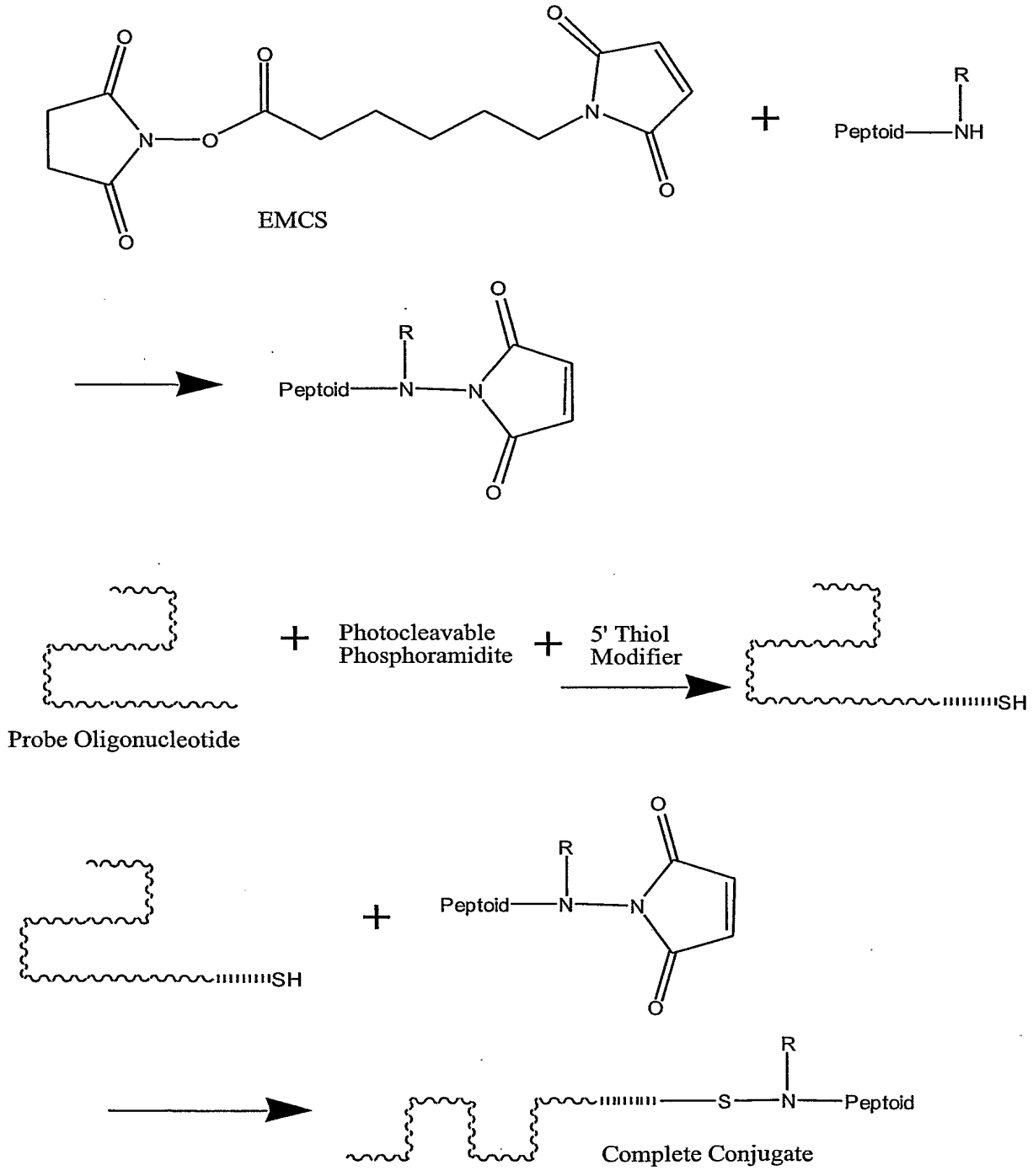
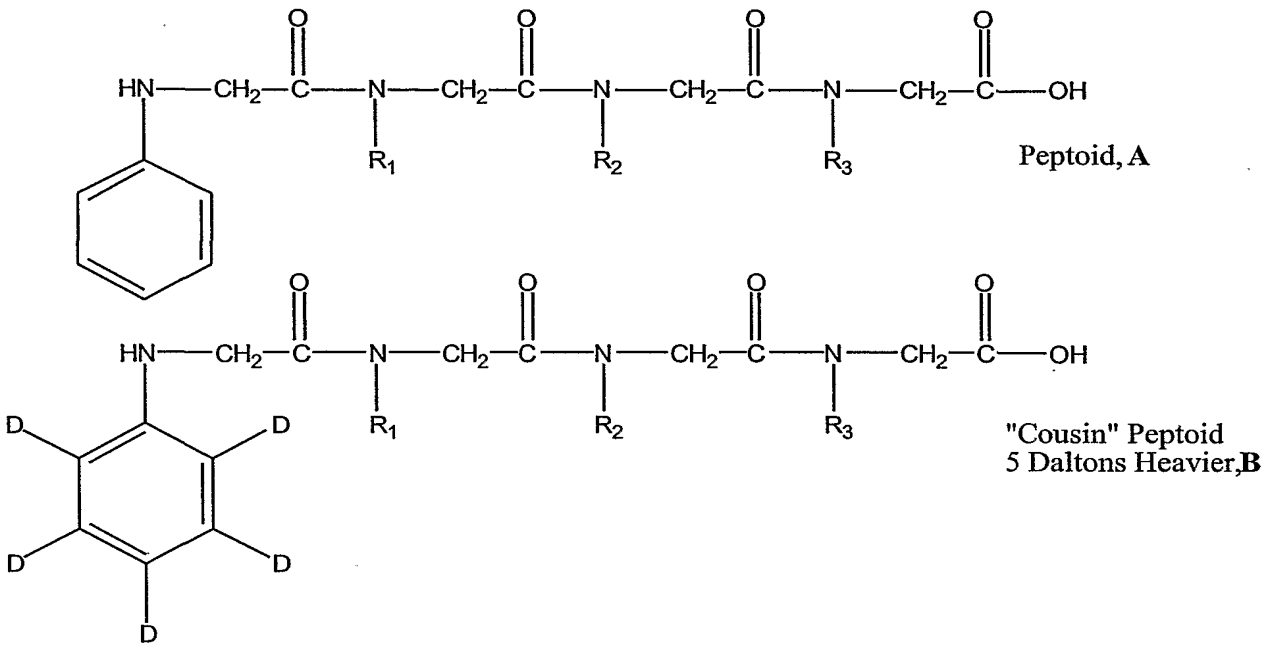

EMCS
Peptoid
NH
R
Photocleavable
Phosphoramidite
5' Thiol
Modifier
SH
Probe Oligonucleotide
S
Complete Conjugate

FIGURE 15


HN—CH2—C—N—CH2—C—N—CH2—C—N—CH2—C—OH
R1
R2
R3
Peptoid, A
D
"Cousin" Peptoid
5 Daltons Heavier, B

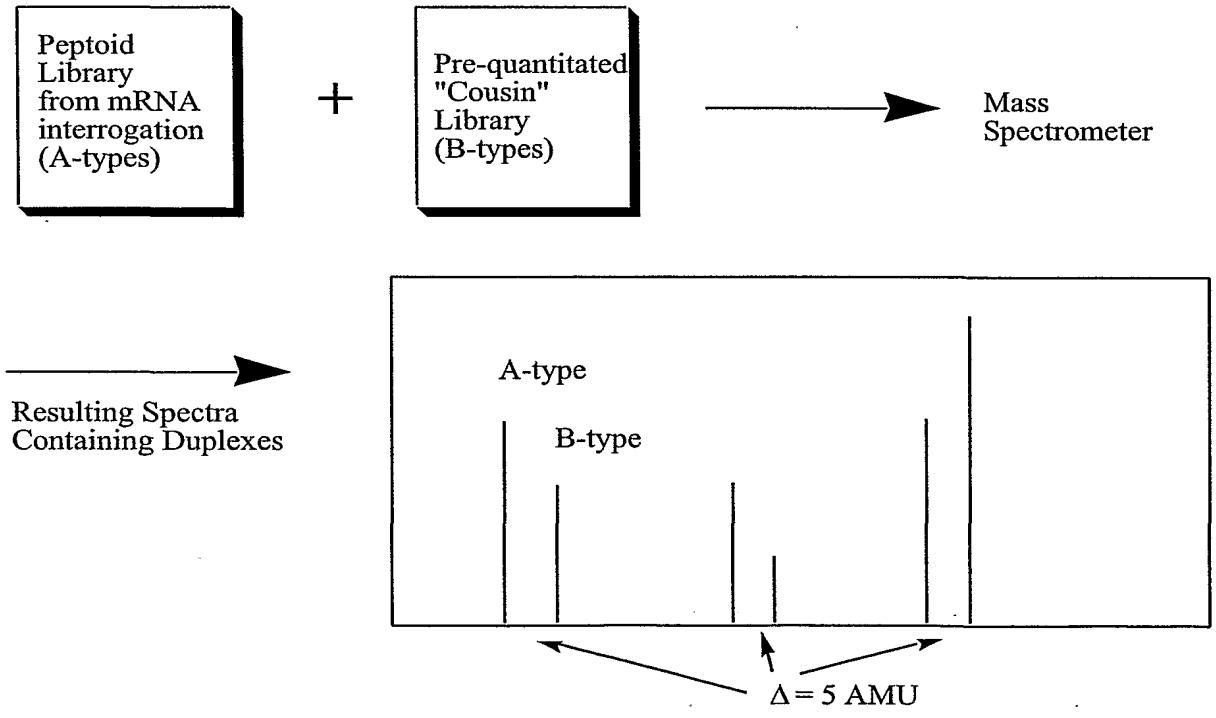
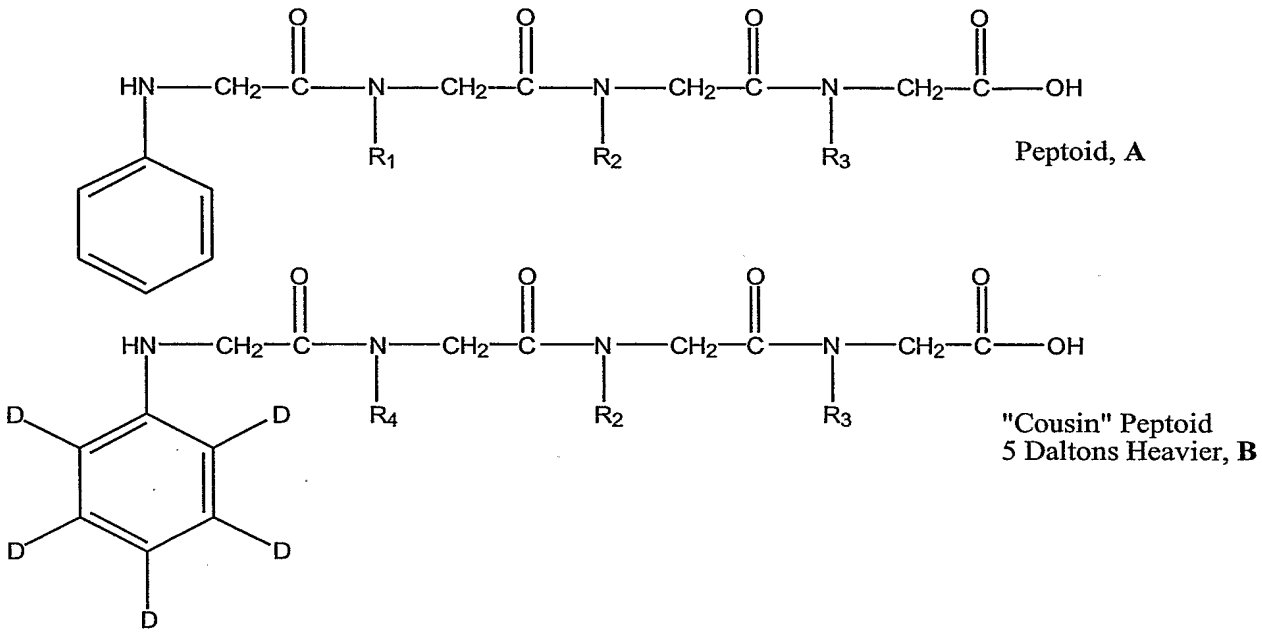

Peptoid Library from mRNA interrogation (A-types)
+
Pre-quantitated "Cousin" Library (B-types)
Mass Spectrometer
Resulting Spectra Containing Duplexes
A-type
B-type
Δ = 5 AMU

FIGURE 16


HN—CH2—C—N—CH2—C—N—CH2—C—N—CH2—C—OH
R1
R2
R3
Peptoid, A
HN—CH2—C—N—CH2—C—N—CH2—C—N—CH2—C—OH
D
D
D
D
D
R4
R2
R3
"Cousin" Peptoid
5 Daltons Heavier, B

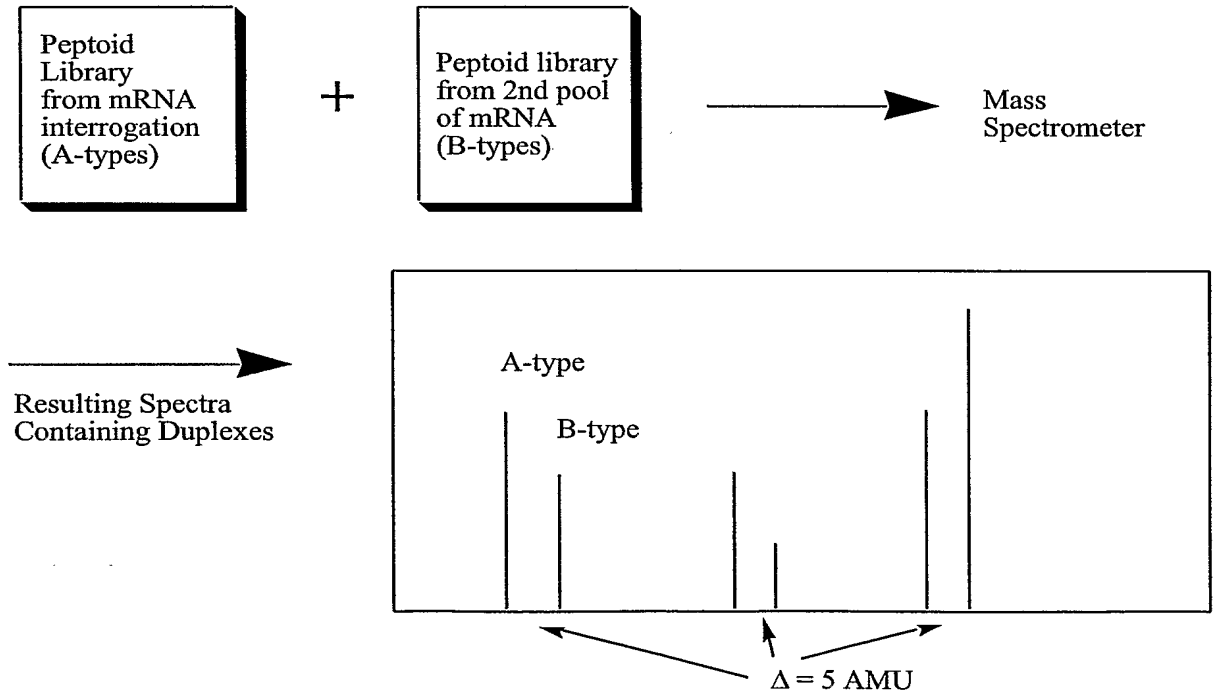

Peptoid Library from mRNA interrogation (A-types)
+
Peptoid library from 2nd pool of mRNA (B-types)
Mass Spectrometer
Resulting Spectra Containing Duplexes
A-type
B-type
Δ = 5 AMU

FIGURE 17

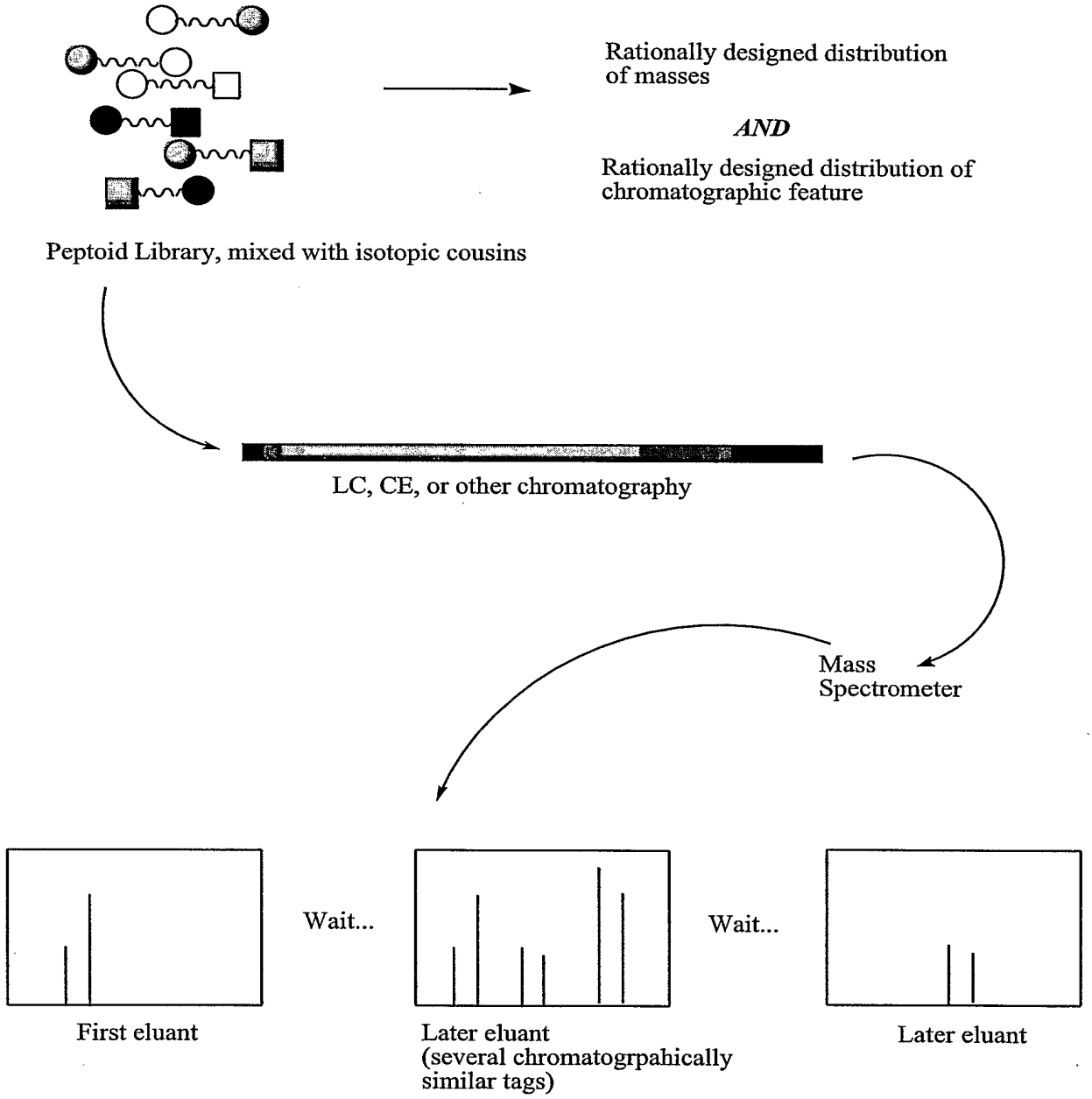
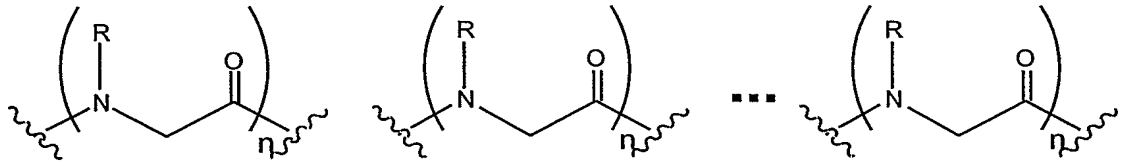

Rationally designed distribution of masses
AND
Rationally designed distribution of chromatographic feature
Peptoid Library, mixed with isotopic cousins
LC, CE, or other chromatography
Mass Spectrometer
Wait...
Wait...
First eluant
Later eluant (several chromatogrpahically similar tags)
Later eluant

FIGURE 18

1) Synthesize and purify 1000 different peptoid oligomer mass tags, of mass 6,000 to 11,000 Daltons.



M=6,000 "pM6000" M=6,005 "pM6005" M=11,000 "pM11000"

This will be performed by a robotic synthesizer on solid phase, with oligomer lengths of up to 40 residues. Molecular weights per residue will be 150-300 Daltons.

2) Synthesize and purify 16,000 different DNA oligos, complementary to the mRNA specie to be detected. Create 16 libraries of 1,000 oligos each.

$AA(NNNNNNNN)_1$, $AA(NNNNNNNN)_2$, ... , $AA(NNNNNNNN)_{1,000}$

$AC(NNNNNNNN)_1$, $AC(NNNNNNNN)_2$, ... , $AC(NNNNNNNN)_{1,000}$

.....

$TT(NNNNNNNN)_1$, $TT(NNNNNNNN)_2$, ... , $TT(NNNNNNNN)_{1,000}$

3) Specifically conjugate oligos in each library to a corresponding peptoid mass tag.

$AA(NNNNNNNN)_1$/pM6000, $AA(NNNNNNNN)_2$/pM6005, ... , $AA(NNNNNNNN)_{1,000}$/pM11000

$AC(NNNNNNNN)_1$/pM6000, $AC(NNNNNNNN)_2$/pM6005, ... , $AC(NNNNNNNN)_{1,000}$/pM11000

.....

$TT(NNNNNNNN)_1$/pM6000, $TT(NNNNNNNN)_2$/pM6005, ... , $TT(NNNNNNNN)_{1,000}$/pM11000

4) Purify DNA/peptoid mass tag products and combine library elements into 16 pools.

FIGURE 19

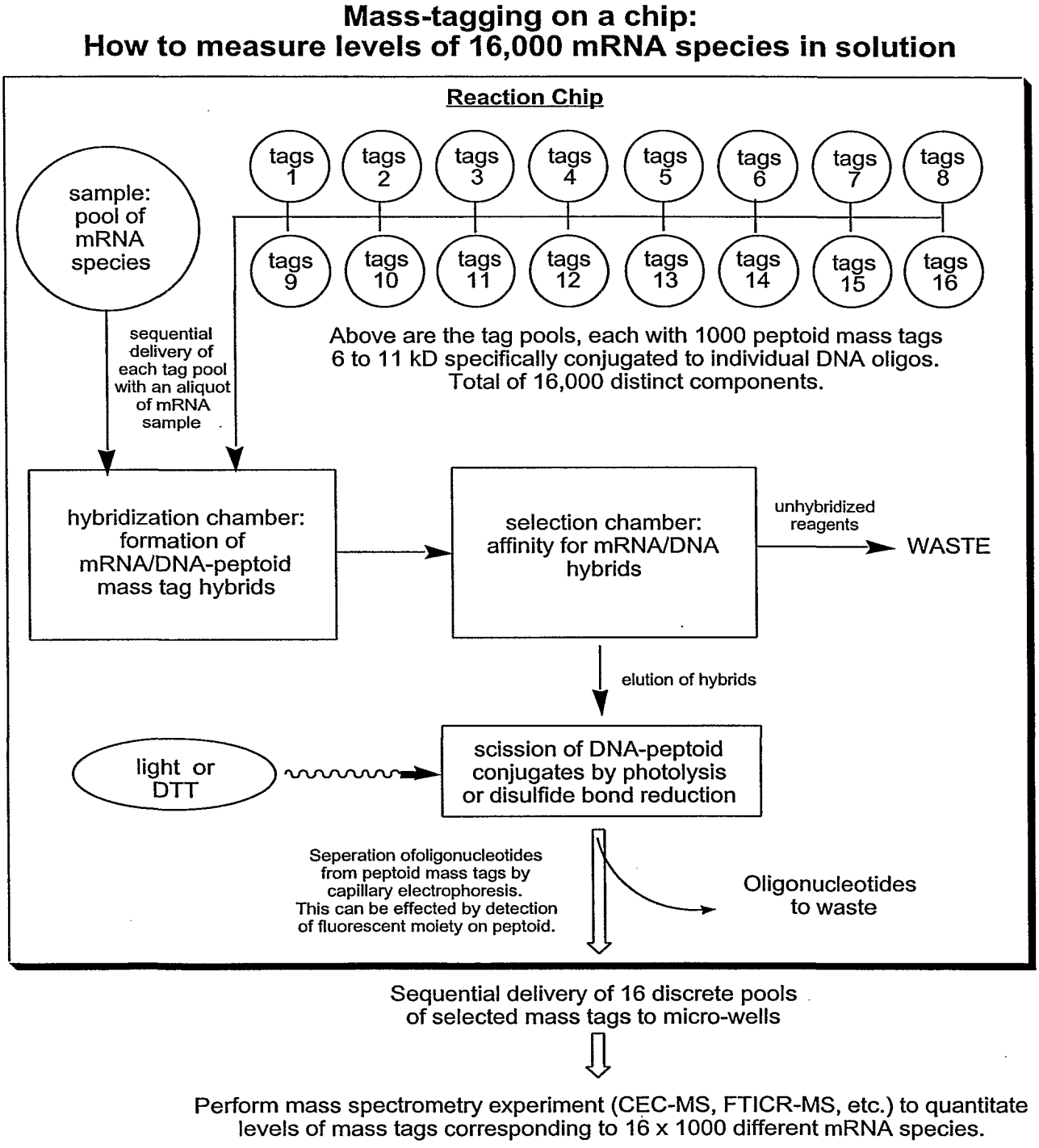
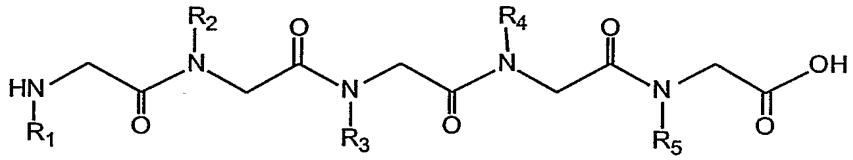
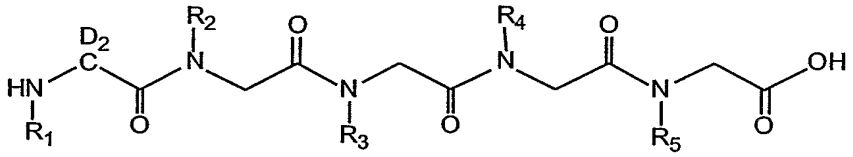

Mass-tagging on a chip:
How to measure levels of 16,000 mRNA species in solution
Reaction Chip
sample: pool of mRNA species
tags 1
tags 2
tags 3
tags 4
tags 5
tags 6
tags 7
tags 8
tags 9
tags 10
tags 11
tags 12
tags 13
tags 14
tags 15
tags 16
sequential delivery of each tag pool with an aliquot of mRNA sample
Above are the tag pools, each with 1000 peptoid mass tags 6 to 11 kD specifically conjugated to individual DNA oligos. Total of 16,000 distinct components.
hybridization chamber: formation of mRNA/DNA-peptoid mass tag hybrids
selection chamber: affinity for mRNA/DNA hybrids
unhybridized reagents
WASTE
elution of hybrids
light or DTT
scission of DNA-peptoid conjugates by photolysis or disulfide bond reduction
Seperation ofoligonucleotides from peptoid mass tags by capillary electrophoresis. This can be effected by detection of fluorescent moiety on peptoid.
Oligonucleotides to waste
Sequential delivery of 16 discrete pools of selected mass tags to micro-wells
Perform mass spectrometry experiment (CEC-MS, FTICR-MS, etc.) to quantitate levels of mass tags corresponding to 16 x 1000 different mRNA species.

FIGURE 20

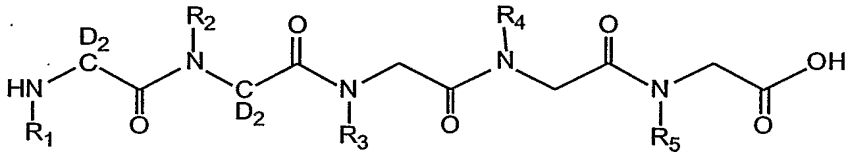
A



B



C



D

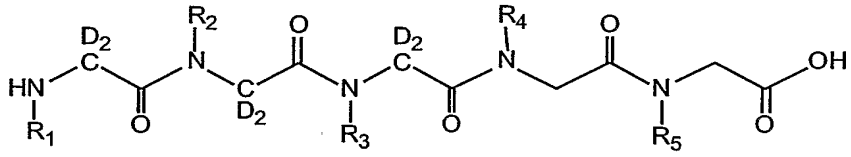


FIGURE 21

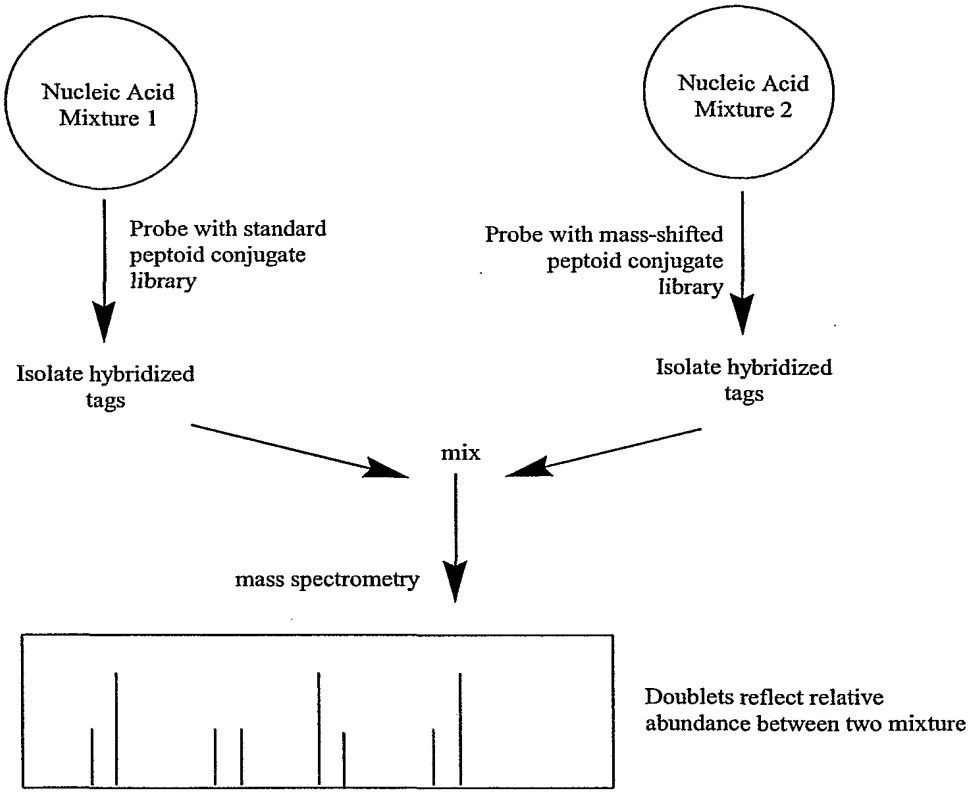

Nucleic Acid Mixture 1
Nucleic Acid Mixture 2
Probe with standard peptoid conjugate library
Probe with mass-shifted peptoid conjugate library
Isolate hybridized tags
Isolate hybridized tags
mix
mass spectrometry
Doublets reflect relative abundance between two mixture

FIGURE 22

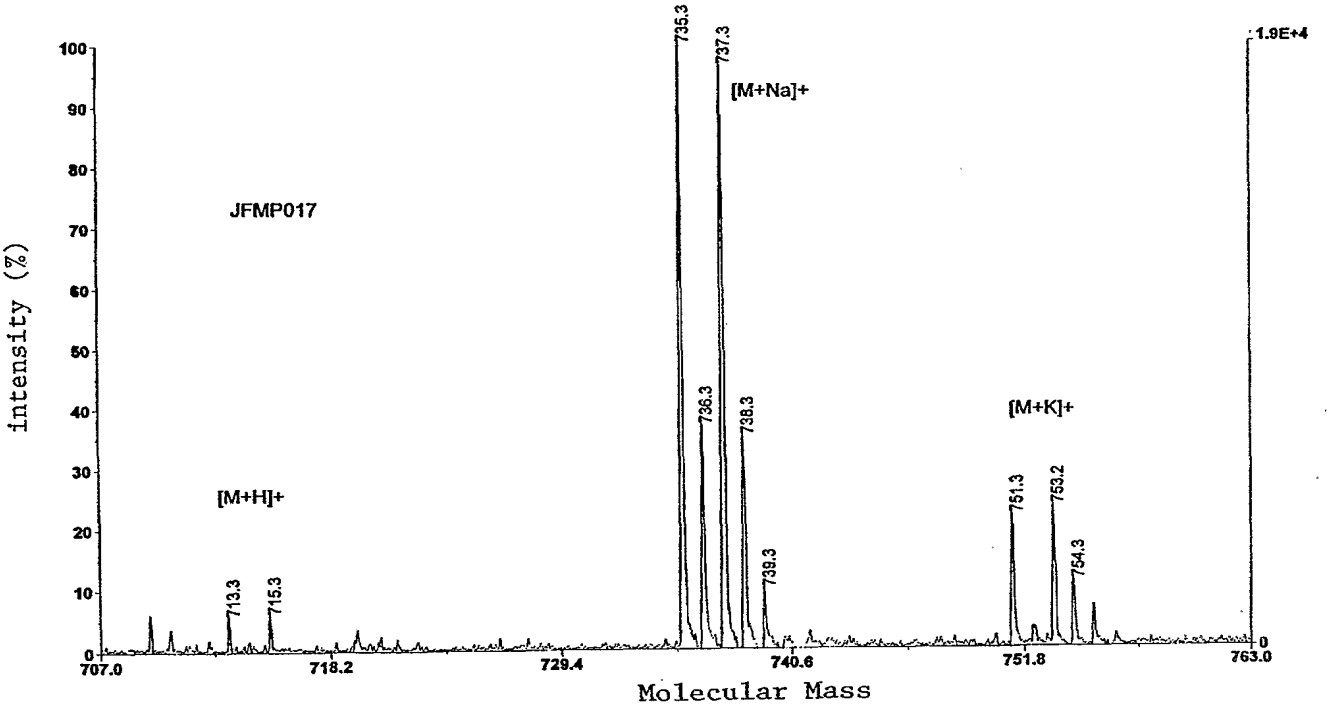

JFMP017
[M+H]+
[M+Na]+
[M+K]+
713.3
715.3
735.3
736.3
737.3
738.3
739.3
751.3
753.2
754.3
1.9E+4
0
intensity (%)
Molecular Mass
707.0
718.2
729.4
740.6
751.8
763.0

FIGURE 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/24021

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12Q 1/68
US CL : 435/6; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.1, 91.2, 183, 287.2, 810; 436/94, 800; 530/350.1, 387.1; 548/303.7; 536/23.1, 24.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST (Files searched: USPAT, UDPGT, EPO, JPO, Derwent); STN (Files: BIOSIS, MEDLINE, CA)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,090,552 A (NAZARENKO et al.) 18 July 2000 (18.07.2000), see entire document.	1-38
A	US 6,075,121 A (SIMON et al.) 13 June 2000 (13.06.2000), see entire document.	1-38
Y	US 5,811,387 A (SIMON et al.) 22 September 1998 (22.09.1998), see entire document.	1-38
A	US 5,714,330 A (BRENNER et al.) 03 February 1998 (03.02.1998), see entire document.	1-38
Y	US 5,681,943 A (LETSINGER et al.) 28 October 1997 (28.10.1997), see columns 5-9.	1-38
Y	US 5,112,739 A (MENEGHINI et al.) 12 May 1992 (12.05.1992), see columns 3 and 4.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2001 (22.09.2001)	27 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Bradley L Sisson Telephone No. (703) 308-0196

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